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Protective effect of *Phyllanthus niruri* leaf extracts on *Saccharomyces cerevisiae* cells subjected to oxidative stress

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Abstract
Phyllanthus niruri has been widely used to treat a number of ailments in traditional medicine. The pharmacological and preventive potential of *Phyllanthus niruri*, such as in urolithiasis, hyperglycemia, hypertension, pain and mild cases of malaria, are well known. The present investigation was designed to study the protective effects of aqueous, methanol and chloroform extract of *Phyllanthus niruri* against oxidative stress and cytotoxicity induced by hydrogen peroxide (H_2O_2) in *Saccharomyces cerevisiae* cells. Cytotoxicity of leaf extracts and H_2O_2 was identified by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sulforhodamine B assay (SRB) and neutral red uptake (NRU) assays. All the three extracts showed protection against H_2O_2 induced toxicity and reduced the loss of cell viability proving the cytoprotective effect of the extracts against H_2O_2 induced toxicity in *Saccharomyces cerevisiae* cells. The aqueous extract rendered maximum protection when compared to that of methanolic and chloroform extract.

Keywords: Oxidative stress, H_2O_2, *Phyllanthus niruri*, *Saccharomyces cerevisiae*, cell viability

Introduction
Oxidative stress describes the level of oxidative damage in a cell, tissue or organ caused by Reactive Oxygen Species (ROS). Factors such as exposure to environmental pollutants, drugs, UV radiation and normal cellular activities cause the production of ROS such as superoxide radical, hydrogen peroxide and hydroxyl radical (Coballase-Urrutia *et al*., 2013) [5]. Oxidative stress results as an imbalance between the formation and neutralization of reactive molecules such as ROS and Reactive nitrogen species (RNS) which can be removed with antioxidants (Kalim *et al*., 2010) [10]. The human body possesses numerous antioxidant defences and repair mechanism against oxidative stress. However, these mechanisms are insufficient to prevent the damage entirely as production of ROS is certain to play important role in tissue damage and loss of function of number of organs and tissues (Gul *et al*., 2013) [7]. A number of *in vitro* studies have demonstrated that oxidative stress induced by chemical oxidants, such as hydrogen peroxide (H_2O_2), leads to cell death (Siddiqui *et al*., 2011) [14]. H_2O_2 has also been reported to induce apoptotic changes, which subsequently lead to death in a variety of cell systems (Sattayasai *et al*., 2013) [13]. Much research has been geared towards the evaluation of plant extracts as prophylactic agents, which offer great potential to inhibit the cell death. The discovery of effective herbs and elucidation of their underlying mechanisms could lead to the development of an alternative and complementary method for cancer prevention and/or treatment (Guilford and Pezzuto, 2008) [6].
Phyllanthus niruri is a widespread tropical plant, commonly found in coastal areas. The extract from aerial sections of *Phyllanthus niruri* was used for the treatment of kidney and bladder diseases, intestinal infections, diabetes and hepatitis B. The species of *Phyllanthus niruri* has demonstrated an antimutagenic and anticarcinogenic property as well as anti hyperlipidemic activity (Junior *et al*., 2012) [9]. The *Phyllanthus* genus contains over 600 species and the extracts of *Phyllanthus niruri* was demonstrated to block the formation of calcium oxalate crystals and stone formation in urolithiasis (Asare *et al*., 2011) [1]. The yeast, *Saccharomyces cerevisiae*, is an excellent model for gaining insights into the molecular basis of human disorders, particularly those resulting from impaired mitochondrial metabolism

(Barrientos, 2003) [3]. Yeasts may provide a particularly useful model for understanding the connections between DNA damage, cell cycle regulation and apoptosis (Weinberger *et al*., 2003) [17]. With this background, the objective of the present study was designed to study the protective effects of aqueous, methanol and chloroform extract of *Phyllanthus niruri* against oxidative stress and cytotoxicity induced by hydrogen peroxide (H_2O_2) in *Saccharomyces cerevisiae* cells.

Materials and Methods

Phyllanthus niruri (Plate 1) (Family: *Euphorbiaceae*) is a perennial herb distributed throughout India. It is useful in the treatment of various ailments.

Plate 1: *Phyllanthus niruri*

Collection of plant sample

The fresh leaves of *P. niruri were* collected, washed in running tap water to remove the surface contaminants and blotted dry between folds of filter paper.

Preparation of methanol and chloroform extracts

One gram of the fresh leaves was homogenized in 10 ml of methanol and chloroform using mortar and pestle. The homogenate was centrifuged at 3000 rpm to clarify the extract. The supernatant corresponding to the concentration of 1mg/µl was used for assay. The supernatant was transferred to a pre weighed beaker and evaporated at 60 ºC protected from light. The residue was weighed and dissolved in dimethyl sulphoxide (DMSO) at a concentration of 5mg/µl. The extracts were tested for their cytoprotective ability.

Preparation of aqueous extract

Aqueous extract was prepared fresh when experiments were performed.

Culturing of yeast cells

YPD medium (10g of yeast extract, 20g of peptone and 20g of dextrose in 1000ml of distilled water, pH 6.5) was prepared and sterilized by autoclaving after aliquoting. The cooled aliquots were stored at room temperature till use and checked regularly for contamination.

Yeast cells were inoculated in the medium on the penultimate day of each assay and the flask was incubated in a controlled orbital shaker at 30ºC overnight. After incubation, the medium was centrifuged at 1000rpm for 15 minutes to pellet out the cells. The cells were then washed with saline and resuspended in a specific volume of assay medium for each assay.

Induction of apoptosis

Cells in the exponential growth phase were harvested and the cell pellet was washed with saline. The pellet was then suspended in saline. Aliquots containing 1×10^6 cells were incubated with H_2O_2 (200mM) and/or leaf extracts (20 mg) at 37 ºC for 1hour. After incubation, the cells were collected by centrifugation, resuspended in saline, and further analyses were performed.

Treatment details

The treatment groups set up for the present study were

T_1: *S. cerevisiae* alone
T_2: *S. cerevisiae* + H_2O_2
T_3: *S. cerevisiae* + aqueous extract of *P. niruri* leaves
T_4: *S. cerevisiae* + H_2O_2 + aqueous extract of *P. niruri* leaves
T_5: *S. cerevisiae* + methanolic extract of *P. niruri* leaves
T_6: *S. cerevisiae* + H_2O_2 + methanolic extract of *P. niruri* leaves
T_7: *S. cerevisiae* + chloroform extract of *P. niruri* leaves
T_8: *S. cerevisiae* + H_2O_2 + chloroform extract of *P. niruri* leaves

Cell viability assays

MTT assay

A total of 1×10^6 cells per Eppendorf microfuge tube were seeded into 96-well plates and exposed for 1 hour to the H_2O_2-plant extract mixture. The cytotoxicity of the drugs was assessed by the MTT assay, based on the procedure of Igarashi and Miyazawa (2001) [8]. MTT (50 µl) was added to the treated wells. The plate was incubated at 37ºC for 3 hours with mild shaking. The cells were resuspended in 200ml of 2-propanol containing 0.04N HCl overnight in the dark. The absorbance was read at 650 nm in a microtiter plate reader (Anthos, Germany).

SRB assay

The fluorescent dye SRB binds to basic amino acid residues in trichloroacetic acid (TCA)-fixed cells to provide a sensitive index of cellular protein content that is linear over a range of cell densities (Skehan *et al*., 1990) [16]. The cell survival was measured as the per cent absorbance at 492 nm, compared to the control (i.e., untreated) cells.

Neutral red assay

The extent of neutral red uptake by the cells was done by the method of Borenfreund *et al*. (1990) [4]. After exposure of the cells to the test agent, the medium was removed. 0.2ml of neutral red containing medium was added per well and incubation was continued for 1 hour at 37 ºC. Cells were then rapidly washed and fixed with 0.2ml solution of 0.5% formalin-1% calcium chloride (1:1) and the neutral red dye in incorporated into the viable cells was released into the supernatant with 0.2ml of solution of acetic acid-50% ethanol. Absorbance was recorded at 540nm with a microtitre plate spectrophotometer.

Results

The cytoprotective effect of *P. niruri* leaf extracts in oxidative stress induced apoptosis on *S. cerevisiae* cells was assessed by cell viability assays such as MTT, SRB and neutral red assay.

Figures 1, 2 and 3 shows the antiapoptotic effect of different extracts of *P. nirui* leaves on the survival of H_2O_2-injured yeast cells. When compared to the apoptotic population, the addition of the extracts to the cells increased the number of surviving cells. Exposure of H_2O_2 reduced the cell number in *cerevisiae* cells. The extracts at a concentration of 20mg play a distinctive role in bringing the cell number to normal.

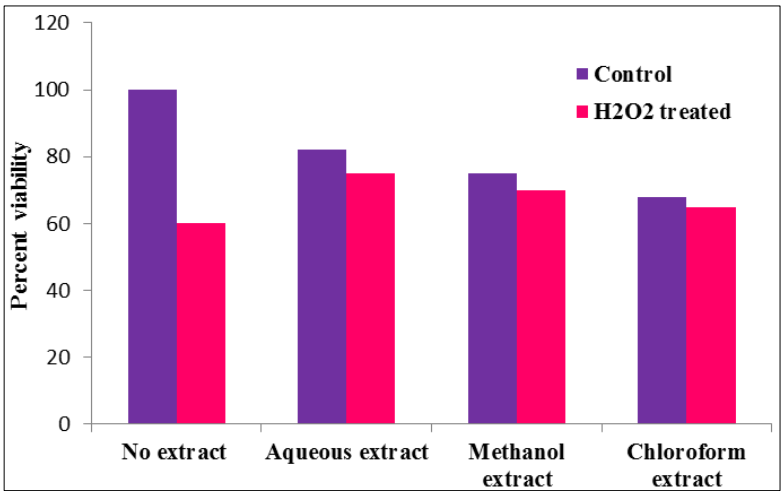

Fig 1: Effect of *P. niruri* leaf extracts on the viability of *S. cerevisiae* cells subjected to oxidative stress as determined by MTT assay. The values of the negative (untreated) control group were fixed as 100% viability and the percentage viabilities in the other groups were calculated relative to this.

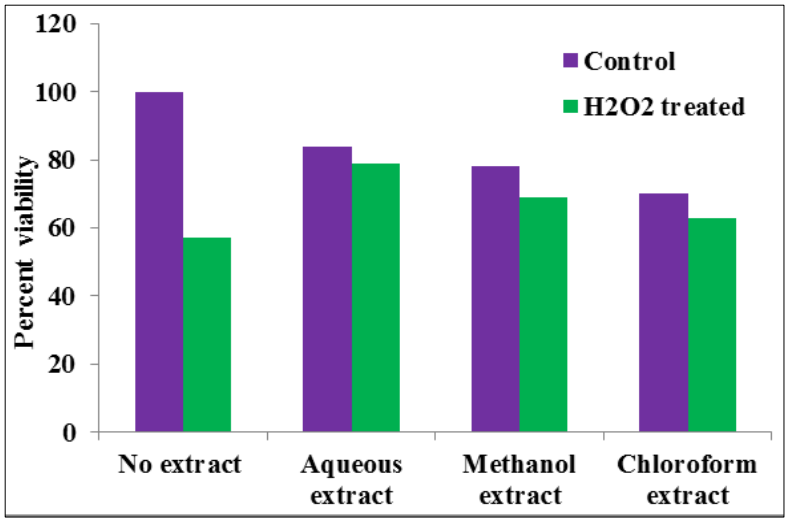

Fig 2: Effect of *P. niruri* leaf extracts on the viability of *S. cerevisiae* cells subjected to oxidative stress as determined by SRB assay. The values of the negative (untreated) control group were fixed as 100% viability and the percentage viabilities in the other groups were calculated relative to this.

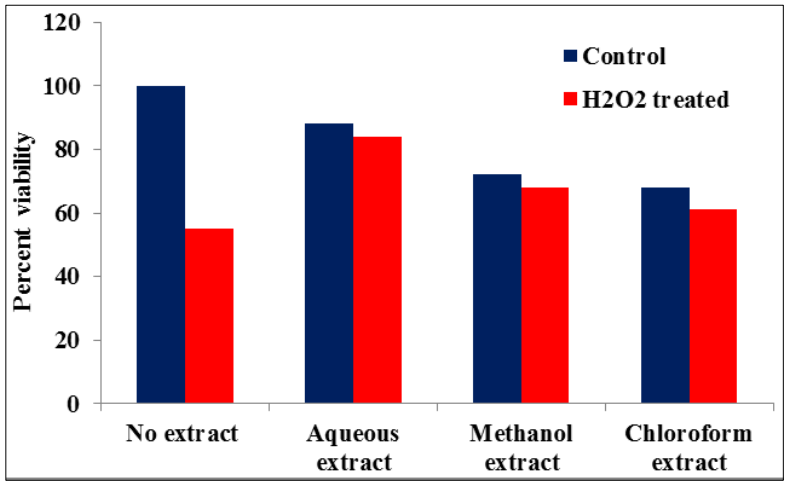

Fig 3: Effect of *P. niruri* leaf extracts on the viability of *S. cerevisiae* cells subjected to oxidative stress as determined by neutral red assay. The values of the negative (untreated) control group were fixed as 100% viability and the percentage viabilities in the other groups were calculated relative to this

Viability assays such as MTT, SRB and Neutral red assays were conducted to determine the cytoprotective effect of *P. nirui* leaf extracts on H_2O_2-induced apoptosis in *Saccharomyces cerevisiae* cells. The viability of the cells increased in the cells treated with *P. nirui* leaf extracts even in the presence of stress inducing agent hydrogen peroxide. The results are indicative that the *P. nirui* leaf extracts possess good antiapoptotic property and is protective against oxidative stress induced cell death. *Zea mays* leaf extracts have a rich source of antioxidants and it can effectively protect the eukaryotic cells (yeast cells) from oxidative stress-induced by hydrogen peroxide (Kiruthika and Padma, 2013) [11]. The apoptosis-inducing effect of the oxidant was effectively counteracted by the presence of methanolic extract of *P. cineraria* leaf extract in oxidative stress induced cell death in *S. cerevisiae* (Bangaruswamy *et al*., 2015) [2]. The methanolic extract of *Majorana hortensis* along with the H_2O_2 showed significant decrease in the apoptotic ratio in *S. cerervisiae* cells when compared to H_2O_2 treated group (Palaniswamy and Padma, 2011) [12]. The methanolic extract of both rhizomes and leaves of *Curcuma amada* did not show cytotoxicity to the *S. cerevisiae* cells and also effectively protected the cells from oxidative damage caused by exposure to H_2O_2 (Sivaprabha *et al*., 2013) [15].

Conclusion

The present study confirm that *P. nirui* leaf extracts are excellent antiapoptotic agents, as validated by their capability to reduce the oxidative stress in *S. cerevisiae* cells that was induced by hydrogen peroxide.

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