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Research Article

PROSOPIS CINERARIA LEAF EXTRACT PROTECT H_2O_2 INDUCED OXIDATIVE STRESS-INDUCED CELL DEATH IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Objective: *Saccharomyces cerevisiae* cells exposed to oxidant damage were used as *in vitro* model to assess the cytotoxic effect and apoptotic events in the presence and absence of the methanolic leaf extract of *Prosopis cineraria*.

Methods: The cytotoxicity assays methyl - tetrazolium, sulforhodamine B (SRB) and lactate dehydrogenase and apoptosis related changes namely, morphological and nuclear changes by various staining techniques (Giemsa, propidium iodide [PI], ethidium bromide [EtBr] and 4',6-diamidino-2-phenylindole), and DNA damage (diphenylamine method) were assessed in *S. cerevisiae* cells with/without leaf extract and oxidant (hydrogen peroxide).

Results: The results of the cytotoxicity assays and apoptotic study showed that H_2O_2 exposure in *S. cerevisiae* cells significantly decreased the viability of the cells. In leaf extract alone treated group, there was no significant reduction in the viability of the cells. When the leaf extract was co-treated with oxidant, there was an increase in the percent viability of the cells indicating that the extract was capable of protecting the cells from oxidative stress.

Conclusion: The apoptosis-inducing effect of the oxidant was effectively counteracted by the presence of methanolic extract of *P. cineraria* leaves.

Keywords: *Saccharomyces cerevisiae,* Oxidative stress, Cytotoxicity, DNA damage.

INTRODUCTION

Oxidative stress is an imbalance between pro-oxidants and antioxidants in the body, and oxidative stress is a complex process [1]. Cells are normally protecting themselves against reactive oxygen species (ROS) damage through the use of intracellular enzymes to maintain the homeostasis of ROS at a low level. However, during environmental stress and cell dysfunction, ROS levels increase dramatically, and cause significant cellular damage in the body [2]. Oxidative stress is a harmful process, which mediates damage to cell structures, including lipids, proteins, RNA and DNA that result in number of diseases [3]. *Saccharomyces cerevisiae* (yeast) is a useful model organism. It is a primitive eukaryote, non-pathogenic fungi and has remarkable similarities to mammalian cells at the molecular and organelle level [4,5]. Previous study proved that the methanolic leaf extract of *Prosopis cineraria* protects the liver slices from hydrogen peroxide induced oxidative stress by enhancing the enzymic and non-enzymic antioxidants levels. Hence, this study was formulated to evaluate the cytotoxicity of the leaf extract in the presence and absence of oxidative stress inducing agent H_2O_2 using *S. cerevisiae* as an *in vitro* model.

METHODS

Preparation of the extract

1 g of fresh leaf sample and 10 ml of methanol were homogenized in a mortar and pestle. The extract was then centrifuged at 2000 rpm for 5 minutes, and the methanol was dried at 60°C protected from light. The resulted residue was weighed and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/20 µl. Free radical scavenging activity was performed with different concentrations, and the dose of 0.15 mg/5 µl of DMSO was optimized. Hence, the same dose was used for assessing the cytotoxicity.

Culturing of *S. cerevisiae* cells

Yeast peptone dextrose (YPD) medium contained 10 g of yeast extract, 20 g of peptone and 20 g of dextrose (pH 6.5) and the total volume of the medium was made up to 1000 ml with water. After aliquoting, the YPD medium was sterilized by autoclaving. The aliquots were cooled and stored at room temperature till use. The medium was regularly checked for contamination.

The medium was inoculated with *S. cerevisiae* cells on the penultimate day of each assay and the flask was incubated in a temperature controlled orbital shaker at 30°C overnight. Overnight grown cultures of *S. cerevisiae* cells were spun down. The cell pellet was washed twice with phosphate- buffered saline (PBS) and resuspended in PBS. Aliquots containing 10^6 cells (counted using Neubauer ruling) were incubated for 1 hr at 30°C with or without H_2O_2 and methanolic leaf extract of *P. cineraria*. A smear was made from the treated cells and used for various staining techniques, whereas the cells in suspension were used to determine the viability.

Treatment groups

1. *S. cerevisiae* alone
2. *S. cerevisiae*+H_2O_2
3. *S. cerevisiae* + methanolic extract of *P. cineraria* leaves
4. *S. cerevisiae* + methanolic extract of *P. cineraria* leaves + H_2O_2

Cytotoxicity assay

The extent of cytotoxicity was determined by the MTT dye reduction assay as described by Igarashi and Miyazawa (2001) [6]. Sulforhodamine assay was performed to assess the extent of cell survival as proposed by Skehan *et al*. (1990) [7].

Lactate dehydrogenase release (LDH) assay

The *S. cerevisiae* cells were treated with the leaf extract in the presence and absence of the oxidant, H_2O_2. A spontaneous control (PBS alone)

and maximum LDH release control (cells in PBS lysed using lysis buffer) were also taken. The cells were then incubated at 37°C for 1 hr and after incubation, the cells were centrifuged. 50µl of the supernatant was taken into a new ELISA reader plate; 50µl of the reconstituted substrate mix (dissolve one vial of the provided substrate mix in 11.4 ml water and add 0.6 ml of assay buffer provided with the kit) was added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by adding 50 µl of stop solution and the absorbance was recorded at 490 nm and the per cent cytotoxicity was calculated as follows:

$$\%\text{Cytotoxicity} = \frac{\text{Experimental-Spontaneous}}{\text{Maximum LDH release}} \times 100$$

Determination of apoptotic changes in the cells

The morphological and nuclear changes associated with apoptosis were studied using various staining techniques. The features of apoptotic cells include shrinkage, condensation of chromatin and cytoplasm, detachment of the cells from the neighboring cells, fragmentation of the nucleus and membrane blebbing.

The Giemsa staining was performed as explained by Chih *et al*. (2001) [8]. The method proposed by Mercille and Massie (1994) [9] was followed to detect the nuclear changes in apoptotic cells, with minor modifications. PI staining was done to monitor the nuclear changes in the apoptotic cells as described by Sarker *et al*. (2000) [10]. The apoptotic cells were detected with 4',6-diamidino-2-phenylindole (DAPI) staining technique as explained by Rashmi *et al*. (2003) [11].

Quantification of DNA fragmentation with diphenylamine

DNA fragmentation is an important hallmark of apoptosis. The percent DNA fragmentation was assessed by the method of Boraschi and Maurizi (1998) [12].

Statistical analysis

Values were expressed as mean ± standard diviation (SD). Statistical difference in mean was analyzed using one way ANOVA and followed by least square mean deviation comparison tests (LSD). $p<0.05$ was considered statistically significant.

RESULT AND DISCUSSION

Assessment of viability and cytotoxicity of *S. cerevisiae* cells

The viability of the cells was analyzed by MTT and sulforhodamine B (SRB) assay. Fig. 1a and b shows drastic decrease in the viability of cells on exposure to hydrogen peroxide, whereas the co-treatment with the methanolic leaf extract of *P. cineraria* reverted the cells from oxidative stress and increased the cell viability.

Our results are in agreement with the observations of Essid *et al*. (2012) [13] who reported that pretreatment of hepatocyte cultures with silibinin protected the cells from cytotoxicity induced by ochratoxin A, Actinomycin D/TNF-α, H_2O_2 and UV as confirmed by MTT test. The viability of *S. cerevisiae* cells decreased upon hydrogen peroxide treatment and this was improved on co-treatment with *Caesalpinia pulcherrima* flower extracts [14]. The methanol extract of *Euphorbia antiquoram* latex milk improved the viability of spleen cells treated with etoposide [15]. Similarly, the hydrogen peroxide treated *S. cerevisiae* cells showed a decrease in viability whereas treatment with *Nytcanthes arbor-tristis* increased the viability of the cells [16]. Treatment of mouse splenocytes with mitogen lipopolysaccharide and lectin stimulated the proliferation of cells. The proliferation was further increased in presence of aerial part extracts (aqueous, ethyl acetate and methanol) from *Cyperus rotundus* [17].

Following the MTT and SRB assay, LDH release assay was performed to confirm the cytotoxicity of the methanolic leaf extract of *P. cineraria* in *S. cerevisiae* cells. The LDH leakage into the medium indicates cell damage [18]. Cells treated with hydrogen peroxide, showed an increase in the LDH release into the medium, which indicated oxidant-induced cell damage. The treatment with the leaf extract counteracted this effect, which was indicated by a decrease in the LDH release as shown in Fig. 1c. Many researchers have reported the influence of plant extract on LDH release in normal cells. Lakshmi (2012) [19] reported that the co-administration of methanolic leaf extract of *Artemisia vulgaris* decreased the LDH release from *S. cerevisiae* cells exposed to hydrogen peroxide. Co-treatment of the *A. vulgaris* leaf extract, artemisinin and dihydroartemisinin showed a decrease in the LDH release in the presence of the oxidant (H_2O_2), thus indicating the protection rendered to the *S. cerevisiae* cells [20]. Methanolic extract of Hawk tea (*Litsea coreana* Levl.var.lanuginose) protected the rats from hepatic damage induced by CCl_4 by decreasing serum LDH levels [21].

The results of the cytotoxicity assays showed that the methanolic leaf extract of *P. cineraria*, by itself, did not cause significant reduction in the viability of *S. cerevisiae* cells, indicating that the leaf extract is not toxic to the yeast cells. In the presence of oxidant, hydrogen peroxide, the leaf extract protected the cells by increasing the viability. Further, to characterize the apoptosis influencing activity of the leaf extract in the presence of oxidant, several staining techniques were carried out.

Apoptosis influencing activity of *P. cineraria*

Morphological changes of apoptosis in S.cerevisiae cells

The characteristic features of apoptotic cells are cell shrinkage, membrane blebbing and cell swelling [22]. These morphological and nuclear changes that resulted by treating the cells with oxidant H_2O_2 in the presence and absence of the methanolic leaf extract of *P. cineraria* were analyzed by Giemsa, EtBr, PI and DAPI staining. Figs. 2-5 shows the apoptotic changes in the cells. The apoptotic ratio was also calculated for each group and the values obtained for all staining methods are shown in Table 1.

There was an increase in the number of apoptotic cells in oxidative stress induced group than the untreated control group, as indicated by an increase in the apoptotic ratio (Table 1). In leaf extract treated group the number of apoptotic cells were reduced, indicating that the extract itself did not show cytotoxicity towards the *S. cerevisiae* cells. When administered along with H_2O_2, the leaf extract caused a marked decrease in the number of apoptotic cells.

The present study falls in line with the previous study of Palaniswamy and Padma (2011) [23] that 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. The nuclear changes in chick embryo fibroblasts by etoposide in the presence and absence of methanolic extract of latex of *Euphorbia antiquorum* proved that the latex imparted complete protection to cells and modulated the apoptotic effects produced by etoposide [24]. Sivaprabha *et al*. (2013) [4] reported that 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 by various staining methods. The thymoquinone compound protected the *S. cerevisiae* cells from H_2O_2-induced damage by decreasing the number of apoptotic cells [25].

Table 1: Apoptotic ratio of various staining techniques in *S. cerevisiae* cells

Staining techniques	Without H_2O_2		With H_2O_2	
	Control	Leaf extract	Control	Leaf extract
Giemsa	0.30	0.36	1.24	0.34
EtBr	0.20	0.38	1.67	0.45
PI	0.37	0.32	1.87	0.52
DAPI	0.25	0.37	1.41	0.38

Saccharomyces cerevisiae: S. cerevisiae, EtBr: Ethidium bromide, PI: Propidium iodide, DAPI: 4',6-diamidino-2-phenylindole

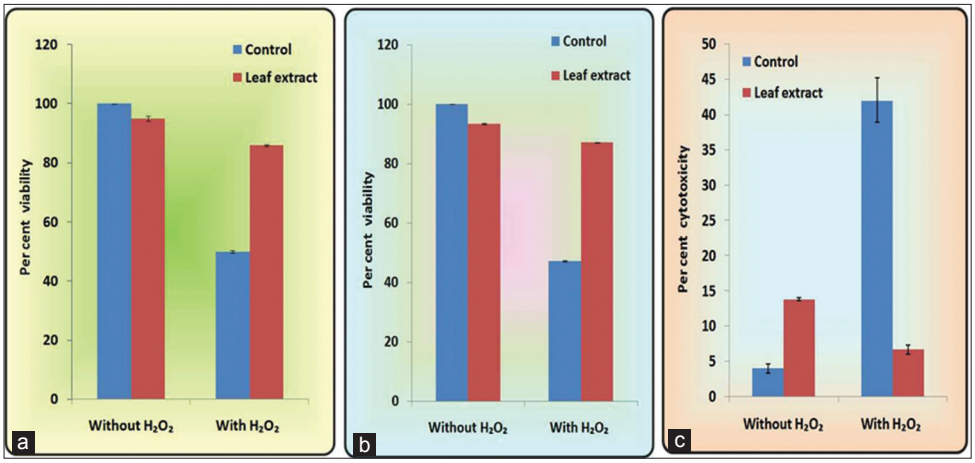

Fig. 1: The effects of methanolic leaf extract of *Prosopis cineraria* on the viability and cytotoxicity of *Saccharomyces cerevisiae* cells, values are mean ± standard deviation of triplicates. The values of the untreated (negative) control group were fixed as 100% and the per cent viabilities in the other groups were calculated relative to this. (a) methyl - tetrazolium assay, (b) sulforhodamine B assay, (c) lactate dehydrogenase release assay

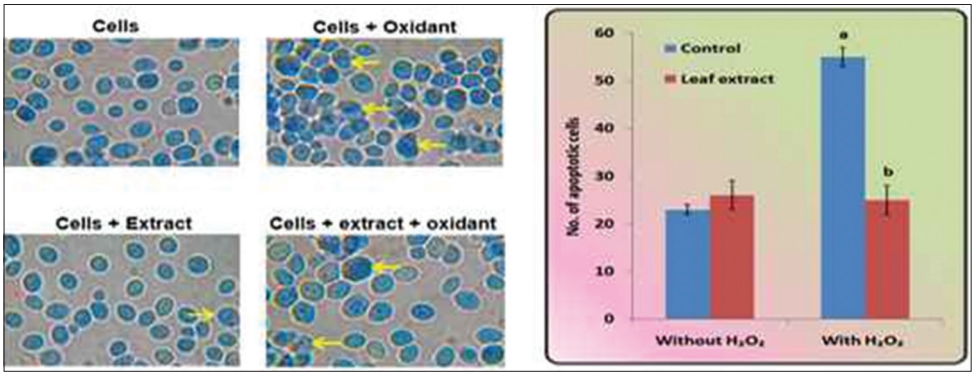

Fig. 2: The morphological changes under the phase contrast microscope in *Saccharomyces cerevisiae* cells by Giemsa staining, arrows indicate the apoptotic cells, values are mean ± standard deviation of triplicates, a - Statistically significant (p<0.05) compared to untreated control, b - Statistically significant (p<0.05) compared to H_2O_2 alone treated group, c - Statistically significant (p<0.05) compared to the respective plant extract treated group

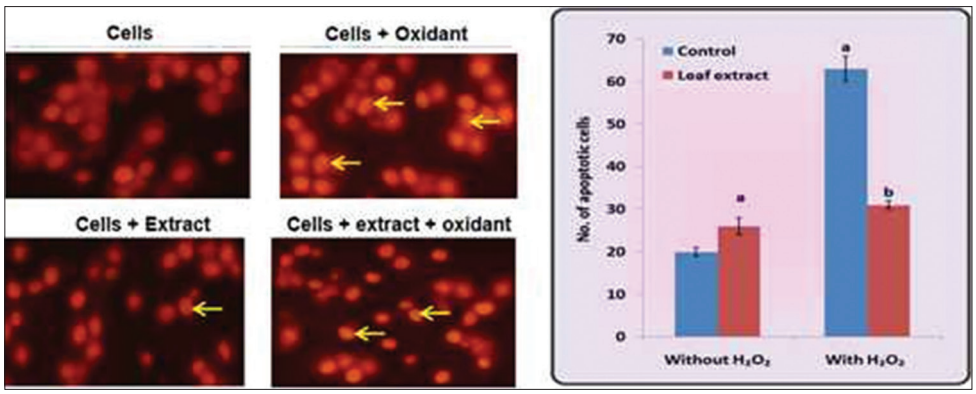

Fig. 3: The fluorescence microscopic images of nuclear changes in *Saccharomyces cerevisiae* cells by ethidium bromide staining, Arrows indicate the apoptotic cells, values are mean ± standard deviation of triplicates, a: Statistically significant (p<0.05) compared to untreated control, b: Statistically significant (p<0.05) compared to H_2O_2 alone treated group, c: Statistically significant (p<0.05) compared to the respective plant extract treated group

Sulekha (2011) [26] has reported that the *Piper bettle* leaf extract protected the *S. cerevisiae* cells from H_2O_2-induced damage by increasing the number of live cells. *Syzygium aromaticum* extract and eugenol significantly reduced the apoptotic events in *S. cerevisiae* exposed to H_2O_2, as evidenced by various stainings [27]. Merlyn (2014) [28] have studied various stainings and proved that the grape skin extract and quercetin protected the *S. cerevisiae* cells from oxidant-induced damage.

DNA fragmentation in *S. cerevisiae*

DNA fragmentation is an important feature of apoptosis. The percentage DNA damage was measured spectrophotometrically using diphenylamine method. The oxidative stress induced by hydrogen peroxide caused a steep increase in the DNA damage, which was drastically reduced in the presence of the *P. cineraria* leaf extract. Thus, these results confirmed that the *P. cineraria* leaf extract was able to protect the *S. cerevisiae* cells

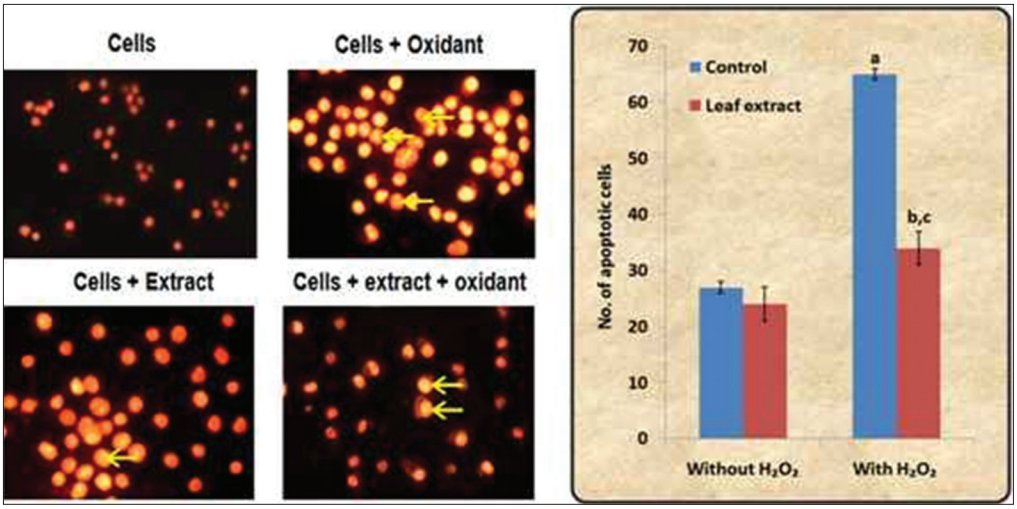

Fig. 4: The fluorescence microscopic images of nuclear changes in *Saccharomyces cerevisiae* cells by propidium iodide staining, Arrows indicate the apoptotic cells, values are mean ± standard deviation of triplicates, a: Statistically significant (p<0.05) compared to untreated control, b: Statistically significant (p<0.05) compared to H_2O_2 alone treated group, c: Statistically significant (p<0.05) compared to the respective plant extract treated group

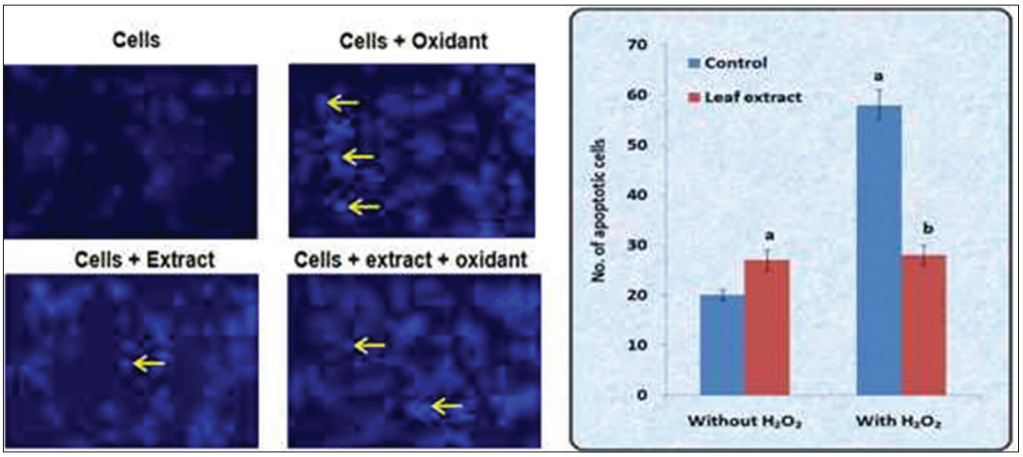

Fig. 5: The fluorescence microscopic images of nuclear changes in *Saccharomyces cerevisiae* cells by 4',6-diamidino-2-phenylindole staining, Arrows indicate the apoptotic cells, values are mean ± standard deviation of triplicates, a: Statistically significant (p<0.05) compared to untreated control, b: Statistically significant (p<0.05) compared to H_2O_2 alone treated group, c: Statistically significant (p<0.05) compared to the respective plant extract treated group

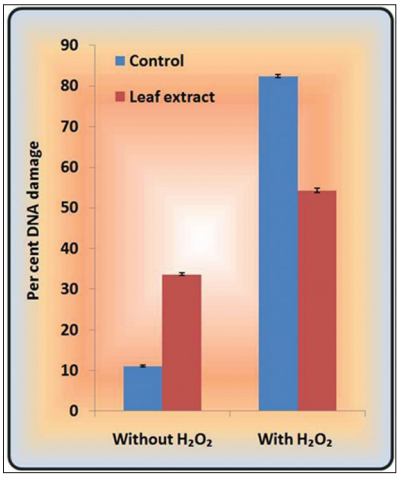

Fig. 6: The effect of methanolic leaf extract of *Prosopis cineraria* on the DNA damage in *Saccharomyces cerevisiae* cells, values are mean ± standard deviation of triplicates

from oxidant induced damage (Fig. 6). The exposure to oxidant caused a significant DNA damage, which was counteracted by the administration of the leaf extract indicating the anti-apoptotic effect of *P. cineraria* leaves.

Diab and Aboul-Ela (2012) [29] showed that pre and post-treatment of date pits (*Phoenix dactyliferal*) aqueous extract significantly restored the DNA damage induced by N-nitroso-N-methyl urea in mice as determined by the DPA assay. A dose dependent increase in DNA fragmentation was observed in Ehrlich Ascites carcinoma cells on treatment with *Withana somnifera* leaf extract by DPA reaction [30]. Chandrashekar *et al*. (2010) [31] showed that the methanolic leaf extract of *Tinospora cordifolia* exhibited DNA protectant activity on oxidative DNA damage in human peripheral lymphocytes. Carbon tetrachloride caused a significant increase in the percent DNA fragmentation whereas methanolic plant extract of *Launaea procumbens* ameliorated the increase induced by CCl_4 [32]. Peritoneal macrophage from mice treated with nicotine showed increased DNA damage and this was reduced by aqueous aerial plant extract of *Ocimum gratissimum* Linn [33]. The observations made in present study clearly indicate that the *P. cineraria* leaf extract exerted a protective effect from hydrogen peroxide induced stress in the *S. cerevisiae* cells.

CONCLUSION

Thus, the study revealed that the methanolic extract of *P. cineraria* leaves is capable of rendering protection against oxidation induced damage. The mechanism exerted by the plant extract in protecting the normal cells from oxidative damage can be probed in future which will be of great significance in modern scientific research as many diseases arise due to oxidative stress.

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