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# Anticancer activity of methanolic extract of *Boerhavia diffusa L*

**Dr. J. Jayachitra*[1] and B. Janani [2]**

[1*]Assistant Professor, Post Graduate and Research Department of Biochemistry,

Rabiammal Ahamed Maideen College for Women – 610001, Tamilnadu, India,

E-mail Id: jayachitra.manikandan@gmail.com

[2] Research Scholar, Post Graduate and Research Department of Biochemistry,

Rabiammal Ahamed Maideen College for Women – 610001, Tamilnadu, India

## Abstract

The present study was carried out to the *in vitro* anticancer activity of methanolic extract of *Boerhavia diffusa L* (Nyctaginaceae) exhibited significant anti hepato cancer activity against a human hepato cancer cell line $HepG_2$ by MTT assay. ***Boerhavia*** which habitually known as '**Punarnava**" that has been classified as "rasayana" herb which possess properties like strengthening life and disease prevention; in other words, providing immunomodulation and hepatoprotection.. *Boerhavia diffusa* extract possess potent anticancer activities.

**Keywords:** *Boerhavia diffusa L*, cell line $HepG_2$ , Anticancer, MTT

## INTRODUCTION

Cancer is pigeonholed by unrestrained splitting up of cells and the capacity of cancerous cells to invade other tissues either by direct growth or by metastasis. Millions of people are diagnosed with cancer every year and many of them die due to the fatal consequences of the disease. Current doctrine states that cancer is a multi-gene, multi-step disease of clonal origin with an altered DNA sequence leading to mutation.Uncontrolled proliferation of these abnormal cells is tailed by a second mutation leading to the insignificantly aberrant stage. Consecutive rounds of mutation and selective expansion of these cells results in the development of a tumor mass **(Momna Hejmadi, 2010**).

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and is the fifth most frequent cancer form and the third most common cause of cancer related deaths worldwide (**Villanueva *et al*.,2011**). Annually about 500,000-1,000,000 new cases of HCC occur and 600,000 persons die due to HCC (**Montalto, *et al*.,2002**). The incidence of



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liver cancer and liver cancer related death varies greatly around the world, depending mostly on geographic and socio-economic prerequisites in different areas of the world. Regions that have higher incidences of viral hepatitis of different types have higher incidence of HCC and the incidence varies with the type of virus hepatitis that is dominating in the area. Over 80% of the endemic viral infection related HCC occur in sub-Saharan Africa and Southeast Asia (**Yang *et al*.,2010**).

Anticancer agents mainly exhibit a preventative or curative role in a damaged system. Under normal conditions, the cells in which the DNA or other components were irreversibly damaged by various causes, undergo programmed cell death (apoptosis) (Lee et al., 2004).

The systematic experimental steps in order to determine the potential cytotoxicity of drug at different concentrations by MTT assay are presented in video form. It is shown that a decreasing absorbance at 540 nm in the cells treated with increasing concentration of the drug in comparison to the control cells without any treatment. A decreased absorbance in the cells treated with drug suggesting cytotoxicity. MTT assay significantly helps the researchers to determine whether any of the test compounds has cell toxicity or proliferative activity (**Alley *et al.*, 1988).**

Medicinal plants have been used as an exemplary source for centuries as an alternative remedy for treating human diseases because they contain numerous active constituents of therapeutic value. The development of microbial resistance to antibiotics has led the researches to investigate the alternative sources for the treatment of resistant strains. Presently 80 percent of the world population relies on plant derived medicines and serves as first line of defence in maintaining health and combating many diseases. pathogens. However, very little information is available on such activity of medicinal plants **(Nameirakpam Nirjanta Devil *et al.*, 2014).**

*Boerhavia diffusa* belongs to the family Nyctaginaceae commonly known as ‘punarnava’ and locally called ‘thazuthama’ which means rejuvenating the cells or renewing the body4 by eliminating excess water from it make it highly advantageous for the kidney and the liver. The plant acts on all important organ systems and rejuvenate them, giving a new life. In India it has a long history of use by indigenous and tribal people, and in Ayurvedic or natural herbal medicine. Ayurveda classified this plant as ‘rasayana’ herb which is said to possess properties like antiaging, reestablishing youth, strengthening youth,

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strengthening life and brain power, and disease prevention like jaundice (**Gaitonde *et al*., 1974**), all of which imply that they increase the resistance of the body against onslaught (**Lal *et al*., 1983**). Pharmacological studies have demonstrated that *B. diffusa* known to possessdiuretic (**Mudgal, 1975**), antifertility (**Chopra, 1969**), antifibrinolytic (**Jana, 2007**), immunomodulatory (**Akah et al., 1993**), antidiabetic (**Shah *et al*., 1983**), antiviral (**Parveen *et al*.,2007**), antistress (**Agarwal *etal*.,2008**), antimicrobial (**Jaiswal, 2010**), antiinflammatory, hepatoprotective (**Ramachandra *et al***., 2011), antioxidant (**Gupta *et al*.,2004**), antiurethritis (**Chandan,1991**) and antimetastatic (**Adesina, 1979**). The main aim of the present study was to evaluate anticancer activities of the methanolic extract of *Boerhavia diffusa*.

## MATERIALS AND METHODS

### Plant Material

The plant material of *Boerhavia diffusa*L leaf used for the investigationwas collected fromin and around the village nearby Thanjavur. The plant was identified and authenticated by **Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Chennai** and a voucher specimen is kept in the herbarium.

### Preparation of the extract

300gm of coarsely ground powder was packed into soxhlet column and extracted with 250ml of 70% methanol for 48 hours (64.5-65.5°C). The extract was filtered and concentrated on water bath at reduced pressure (bath tem 50°C) to syrup consistency (yield: 15%). Then the dried extract was stored in air tight container for further use..

### MTT assay

#### *In vitro* in liver cancer Hep G2 cells

#### Study design I: Human liver Cancer Hep G2 cells

#### $IC_{50}$ determination

MTT assay was used for cytotoxicity testing in MCF-7 cells, for *B.diffusa* leaf extract (10, 25, 50, 75, 100, 250 and 500 µg/ml) $IC_{50}$ values were calculated in a dose dependent manner.

#### Cell counting

#### Reagents and equipments



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- 0.04% Trypan blue
- Phosphate-buffered saline (PBS)
- Hemocytometer
- Inverted microscope

**Procedure**

Human Liver cancer HepG2 cells suspension was mixed gently and an aliquot was added to the trypan blue solution (100 µl cell suspension: 100 µl dye) and was then counted in a haemocytometer.

- Calculations

Total no of viable cells = $A \times B \times C \times 10^4$

Total dead count = $A \times B \times D \times 10^4$

Total cell count = Viable cell count × Dead cell count

% of cell viability = Viable cell count × 100 /Total cell count

Where,

A = Volume of cells

B = Dilution factor in trypan blue

C = Mean number of unstained cells

D = Mean number of dead cells or stained cells $10^4$ is the conversion factor for 0.1 $mm^3$ to 1ml

**MTT assay**

The cytotoxicity of *B.diffusa* leaf extract in Hep $G_2$ cells was determined by the method of (**Mosmann, 1983**). MTT is cleaved by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. This formation production is proportionate to the viable number of cells and inversely proportional to the degree of cytotoxicity.

**Principle**

MTT assay, the present yellow tetrazolium salt was metabolized by NAD-dependent dehydrogenase (in active mitochondria) to form a dark blue formazan product)

**Reagents**

- 0.5 g/L MTT
- Dimethyl sulfoxide (DMSO)



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- 96 well plate
- Microplate reader (Bio-Rad)

**MTT stock solution**

MTT (50 mg) dye was dissolved in 10 ml of PBS. After vortexing for 1 min, it was filtered through 0.45 micro filters. The bottle was wrapped with aluminum foil to block the light, as MTT is light sensitivity. The preparation was stored at 4ºC.

**Procedure**

The cells were plated separately in 96 well plates at a concentration of $1\times10^5$ cells/well. After 24 hours, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37°C. After starvation, cells were incubated in a $CO_2$ incubator at 37 ºC at 24 h. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/mL) was added and incubated for 4 hours at 37ºC in a $CO_2$ incubator. The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrical absorbence of the purple, blue formazan dye was measured in a microplate reader at 570 nm (BioRad 680). The results are expressed in the percentage of stable cells with respect to the control. The half maximal inhibitory concentration ($IC_{50}$) values were calculated and the optimum doses were analyzed at different time period.

$$\text{Inhibition of cell proliferation (\%)} = \frac{\text{Mean absorbence of the control} - \text{Mean absorbence of the sample}}{\text{Mean absorbence of the control}} \text{ X } 10$$

The medium effective dose ($IC_{50}$) is the amount of samples able to inhibit cell proliferation by 50%, which was calculated graphically for each well proliferation curve.

**Trypan blue and colony survival assay**

Cell feasibility was measured by the capacity of living cells to eliminate tryphan blue vital dye. Cells were seeded in 96-well micro plates at a concentration of $10^4$ cells / well were treated within the presence and absence of *B.diffusa* leaf extractfor 24 h. After this, the Hep G2 cells were trypsinzed from the micro plates, combined with any moving cells prevailing in the media and pelleted by centrifugation at 1000 g for 10 min at 4 ºC. Cells were washed twice with PBS and tryphan blue was added at a final concentration of 0.2%. Living cells will be counted in a haemocytometer and articulated as the % of the



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whole count in vehicle control. A dose dependent study was accepted for *B.diffusa* leaf extract to find out maximum inhibition.

## RESULT

### CYTOTOXICITY EFFECT OF *B.DIFFUSA* L

Assay: MTT

Concentration: 100 µg/ml

Cell line: HepG2

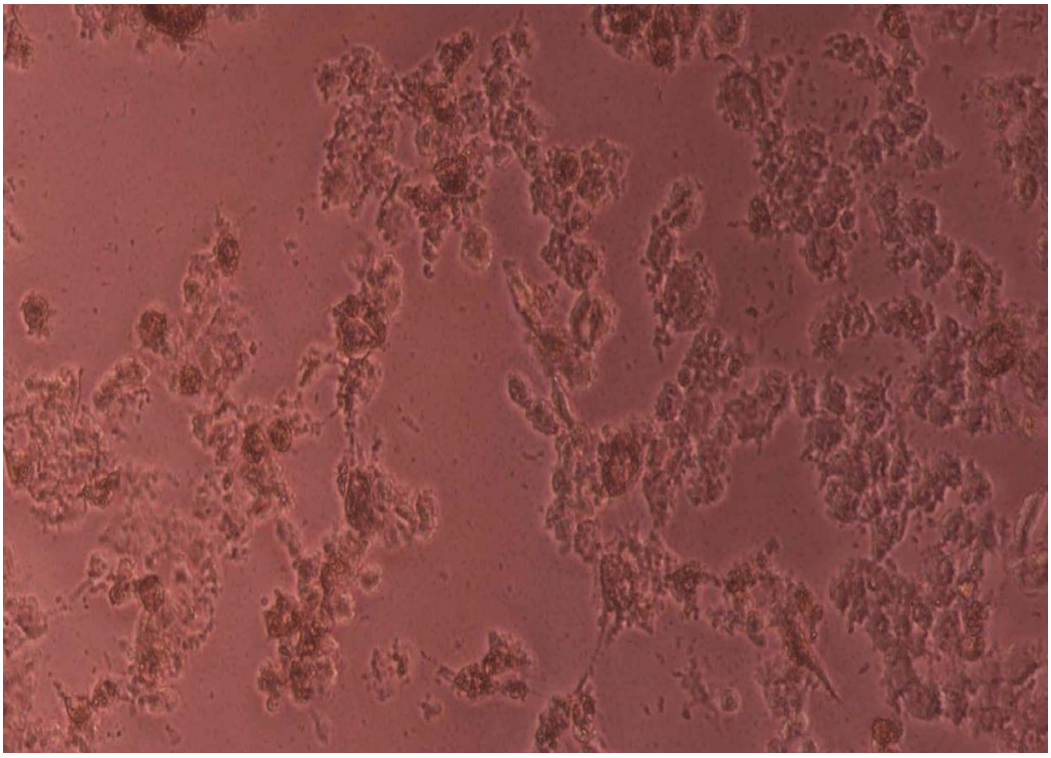


**Fig 2: Control**



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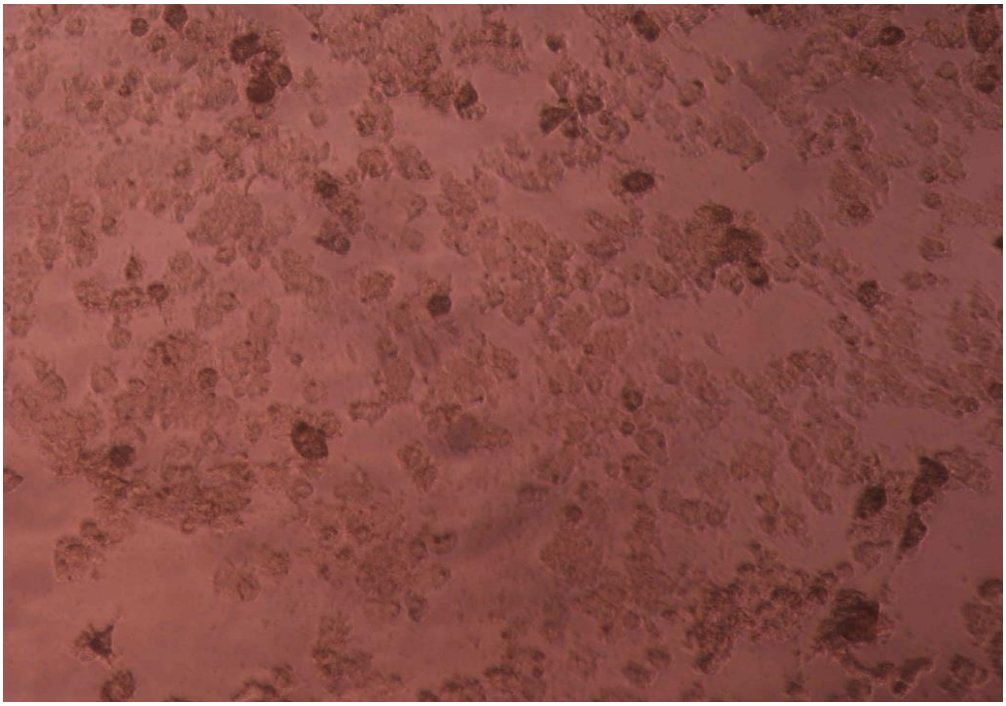


**Fig 3: Cell cytotoxicity of aqueous extract of *Boerhavia diffusa* leaves**

**Effect of *Boerhavia diffusa* on cell viability, growth of Hep $G_2$ cells in a dose dependent manner**

Hep-G2 is an immortal cell line which is consists of human liver carcinoma cells. They can secret many plasminogen proteins; stimulated by human growth hormone. Cancer is a deadly disease; more than 100 types of cancer affects humans. Treating cancer is so painful process as they involve chemotherapy and radio therapies. Phytochemicals does many works in our body. Based upon their work it can also be anti-cancerous agent for various types of cancer. Plant source for treating cancer may reduce painful outcomes unlike from chemotherapy **(WHO, 2014).**

Various preliminary studies (in vitro as well as in vivo) with crude plant extract and its various purified fraction have shown anticancer activity against pulmonary metastases **(Leyon *et a*l., 2005). Bharali et al., (2003)** demonstrated its chemopreventive action against 7, 12- dimethyl benz(a) anthracene (DMBA) induced skin papillomagenesis and **Manu *et al*., (2007)** demonstrated the protective activity of the hydro-alcoholic extract of the plant against gamma radiation induced damage. **Sreeja and Sreeja, (2009)** observed the antiproliferative



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and antiestrogenic properties of methanol extract of Boerhaavia diffusa in MCF-7 breast cancer cell lines suggesting its therapeutic role in estrogen induced breast cancers.

**Mersch-Sundermann *et al*., (2004**) reported that the Gloriosa superba is an alkaloid plant containing a large amount of alkaloid components like colchicine and gloriosine. This study involves anti-cancer examination on Hep-G2 cells (human liver cancer cells) using phytochemical extract obtained from G. superba tuber. G. superba tubers were identified and collected. The solvent which has the highest antioxidant property were preceded to the anti-cancer test against Hep-G2 cells (human liver cancer cells) by MTT assay. The cell death percentage of Hep-G2 cells were calculated from cell viability obtained by MTT assay. Triplicate values were obtained. From the triplicate value the mean value was determined to obtain the average value for each concentration 5 µg, 10 µg, 25 µg, 50 µg, 100 µg. The higher concentration of 100 µg has the lower viable rate and 50% inhibition of viability (IC50) was determined graphically. Death rate of cells indicated the cancer inhibition rate which is due to Hep-G2 cells failed to retain the viability. Inhibition rate of 100 µg has the higher inhibition range of 54.3%. Death rate of Hep-G2 cells may be due to various reasons like protein binding, DNA replication interaction, receptor binding inhibitors, etc. this current study shows the G. superba tubers has the potential to inhibit the growth of Hep-G2 cell.

**Shrivastava *et al.* (2009)** evaluated and compared the cytotoxic activity of ethanolic extract of *B. diffusa* roots and leaves with standard anticancer drug, methotrexate, by MTT assay and with trypan blue dye exclusion method. *In vitro* screening of the extract of *B. diffusa* indicated the crude fraction appeared to be cytotoxic against tumor cells. A dose dependent cytotoxic effect of root and leaf extract was compared with methotrexate. Methotrexate showed almost 40% cell death at a concentration of 200nM, whereas crude ethanolic extract of root showed almost 30% cell death at concentration of 200µg/ml; alkaloid fraction and leaf extract each showed 40% cell death at 300µg/ml. This showed that the plant has promising cytotoxic activity

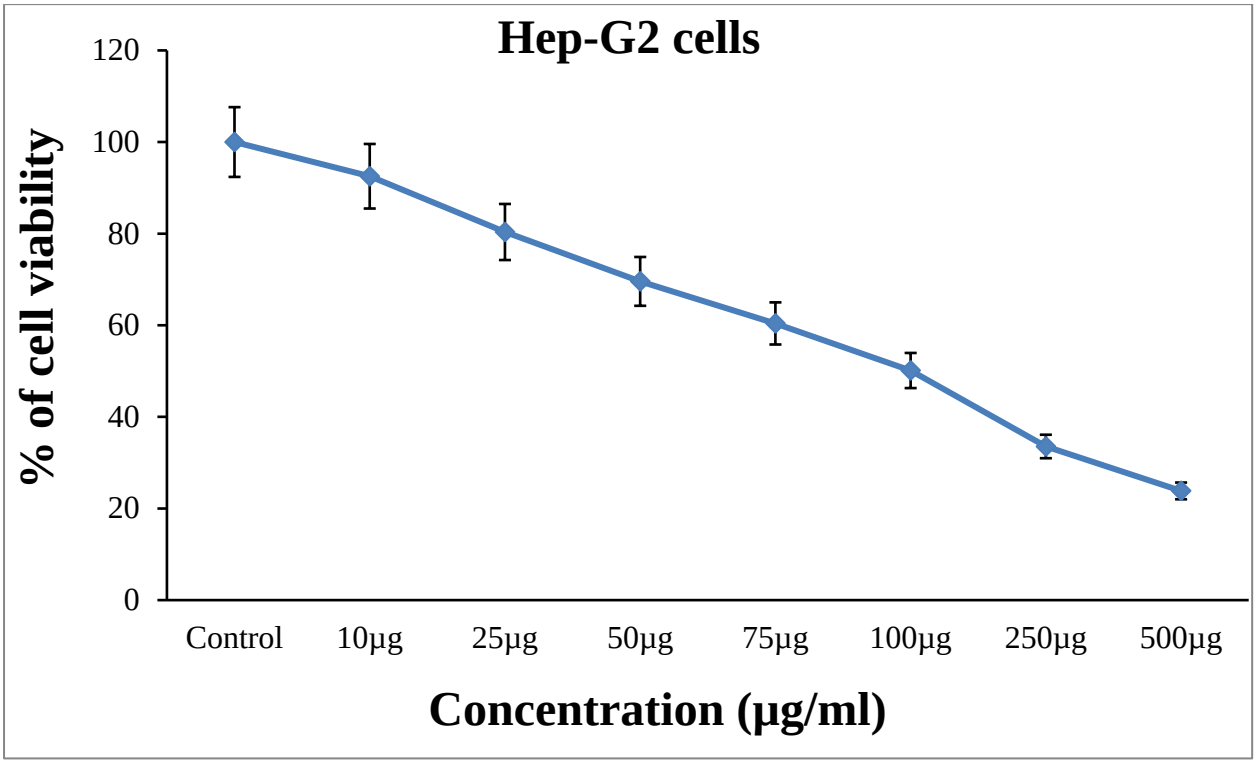
**Fig 4: Effect of *Boerhavia diffusa* on morphological characteristics of Hep $G_2$ cells was assessed by MTT assay.**



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Morphological changes in vehicle control and *Boerhavia diffusa* treated Hep $G_2$ cells for 24 h. The images captured by light microscope (Nikon, Eclipse TS100, Japan) showing (a) intact morphology of vehicle control Hep $G_2$ cells, show *Boerhavia diffusa* treated with Hep $G_2$ cells at different concentration (10, 25, 50, 75, 100, 250 and 500 µg/ml) respectively. Values were presented as mean ± SD of three independent experiments (one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT).

The cytotoxicity effect of *Boerhavia diffusa* in Hep $G_2$ cells has been assessed by MTT assay. Figure 4 shows the % of viable cells was significantly decreased with respect to treatment of *Boerhavia diffusa* with increasing concentrations after 24h. The MTT assay indicated that cell viability decreased to 92%, 78%, 72%, 63%, 50%, 38%, and 29% when cells were exposed to *Boerhavia diffusa* at concentrations of 10, 25, 50, 75, 100, 250 and 500 µg/ml respectively. 50% of viable cells were observed at 100 µg/ml on Hep$G_2$ cells at 24 hrs. From this observation the $IC_{50}$ values of *Boerhavia diffusa* were considered as 100 µg. The survival of Hep $G_2$ cells decreased significantly in a concentration dependent manner with an $IC_{50}$ (the concentration causing 50% growth inhibition) value at 100 µg/ml. There is complete destruction of cells above and 500 µg/ml. A change in cell morphology with a decrease in the cell number was observed in cells treated with 500 µg/ml concentration of *Boerhavia diffusa* (Figure 4). In contrast, no change was formed in the cell morphology was observed with the



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normal Hep $G_2$ cells under the same conditions. It is likely that *Boerhavia diffusa* 100 µg/ml concentration affected the Hep $G_2$ cells, whereas the induced anti-proliferative responses were observed in the Hep $G_2$ cells.

## CONCLUSION

. *Boerhavia diffusa* L. is a plant which is extensively used in folk medicine and little attention has been given to the characterization of secondary metabolites other than rotenoids and alkaloids. Various chemical constituents observed in the phytochemical profiling of *B.diffusa* suggests their significance in pharmacological actions. Study also recommended the species as a plant of phytopharmaceutical importance due to the presence of of biologically active compounds that may serve as candidates for new drugs in the treatment and prevention of many livestock diseases. Anticancer activity showed better results in both the cell lines Hep $G_2$. To rationalise use of the plant however, more work needs to be carried out at molecular level.

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