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Research Article

Apigenin reduces cell viability of Human epidermoid cancer cells (Hep-2)

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ABSTRACT

The aim of the present study is to explore the cytotoxic potential of apigenin in Hep-2 cells. The mechanistic pathway for its cytotoxic potential was analyzed using MTT assay (cytotoxicity), DNA fragmentation (agarose gel electrophoresis), Nuclear damage (DAPI Staining), ROS generation ability (DCFH-DA method), apoptosis inducing potential (dual staining) and expression of apoptosis and angiogenic proteins (Western blotting). The overall findings of the present study suggest that apigenin might have reduced the cell viability of Hep-2 cells through its apoptotic and anti-angiogenic potential.

Keywords: Apigenin; apoptosis; cell viability; Hep-2

INTRODUCTION

Apigenin (4′, 5, 7 trithydroxyflavone), a natural flavonoid is present in several fruits and vegetables such as parsley, onions, orange and wheat products (Rithidech, et al., 2005). Extensive studies documented that a diet rich in apigenin products is associated with decreased risk of heart diseases, neurological disease, allergies and several types of cancer (Tatsuta, et al., 2000). It has been reported that apigenin induces autophagy in leukemia cells (Jayasooriya, et al., 2012). Apigenin exhibited renal protective effect against cyclosporine induced renal damage in rats (Chakravarthi, et al., 2009). Recent studies explored the anti-inflammatory, antioxidant and anticancer potential of apigenin in experimental animal models (Fuchs & Milbradt, 1993; Romanova, et al., 2001; Singh, et al., 2004). Apigenin also exhibited antimutagenic, antiviral and anti-cell proliferative potential in various cancer cell line (Hussain, et al., 2000). Apigenin significantly inhibited the activation of NFκB, which plays a pivotal role in regulating cell proliferation, cell cycle arrest and apoptosis (Hastak, et al., 2003). Apigenin suppressed skin tumor formation in UV-B light induced skin carcinogenesis (Birt, et al., 1997). Apigenin significantly prevented the lung and adenocarcinoma metastasis (Chunhua, et al., 2013). Previous studies from our laboratory demonstrated the chemopreventive and the proapoptotic potential of apigenin in DMBA induced oral carcinogenesis (Silvan, et al., 2011; Silvan & Manoharan, 2013).

The present study was designed to explore the cytotoxic potential of apigenin in Hep-2 cells. The mechanistic pathway for the cytotoxic potential of apigenin was explored with the help of MTT assay (cytotoxicity), DNA fragmentation (agarose gel electrophoresis), Nuclear damage (DAPI Staining), ROS generation ability (DCFH-DA), apoptosis inducing potential (dual staining) and expression of apoptosis and angiogenic proteins (Western blotting). The molecular structure of apigenin is given in figure 1.

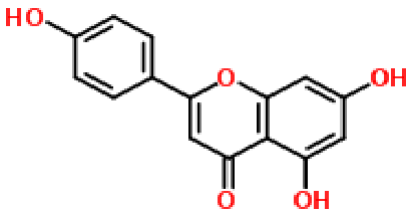

Figure 1: Molecular Structure of Apigenin

MATERIALS AND METHODS

Hep-2 cell line and its maintenance

Hep-2 cell line, purchased from the National Centre for Cell Science (NCCS), Pune, India, was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % Fetal bovine serum (FBS) with 100 U/ml penicillin-G and 100 U/ml streptomycin. Cells were maintained in 5 % CO_2 incubator at 37°C and the experiments were carried out after confluence stage was attained. The experimental work was done in the Cell culture Laboratory, Pondicherry Centre for Biological Science, Pondicherry, India.

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Drug exposure

Hep-2 cells were exposed to various concentrations of apigenin (10-250 µg/ml) and incubated at 37 °C in CO_2 incubator for 24 hours.

Methods

MTT assay, according to the method of Mosmann, (1983), was utilized to find out the efficacy of apigenin on Hep-2 cell viability. The effect of apigenin on the generation of intracellular ROS was assessed according to the method of Pereira, et al., (1999) using a non fluorescent probe 2', 7'- dichlorodihydrofluorescein diacetate (DCFH-DA). The apoptosis inducing potential of apigenin in Hep-2 cells was measured using DAPI staining according to the method of Arung, et al*.,* (2009) as well as by acridine orange/ethidium bromide dual staining according to the method of Baskic, et al., (2006). Apoptotic DNA fragmentation was analyzed in apigenin treated Hep-2 cells, according to the method of Herrmann, et al., (1994). Western blotting is employed to detect the expression of p^{53} Bcl-2, MMP-2 and MMP-9 in apigenin treated Hep-2 cells and untreated Hep-2 cells.

Statistical analysis

Values are expressed as Mean ± Standard deviation (SD). Statistical comparisons were performed by one-way analysis of variance followed by Duncan's Multiple Range Test. The results were considered statistically significant if the *p* values were less than 0.05.

RESULTS

Measurement of cell viability

Figures 2 and 3 illustrate the percentage of cell viability using MTT assay and morphological changes in untreated and apigenin (25 – 200 µg/ml) treated Hep-2 cells. We observed that apigenin at a concentration of 50 µg/ml reduced 50 % cell viability of Hep-2 cells (i.e. IC_{50} = 50 µg/ml).

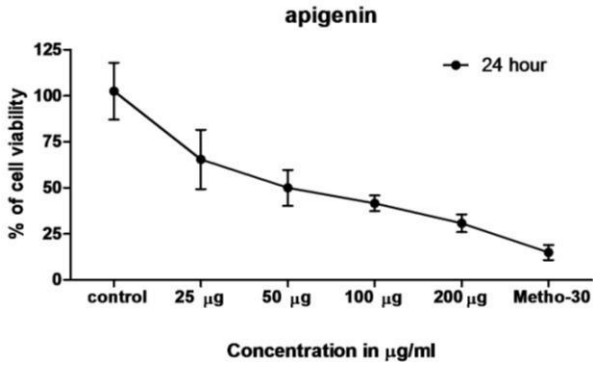

Figure 2: Effect of apigenin on Hep-2 cell viability, measured by MTT assay for 24 hours

Measurement of intracellular ROS

The effect of apigenin on ROS generation in the Hep-2 cells using DCFH-DA staining is shown in figures 4 and 5. Apigenin at a concentration of 50 µg/ml stimulated the generation of ROS than the rest of the doses in the Hep-2 cells. This was further substantiated with higher DCF (green) fluorescence in apigenin treated Hep-2 cells than untreated Hep-2 cells.

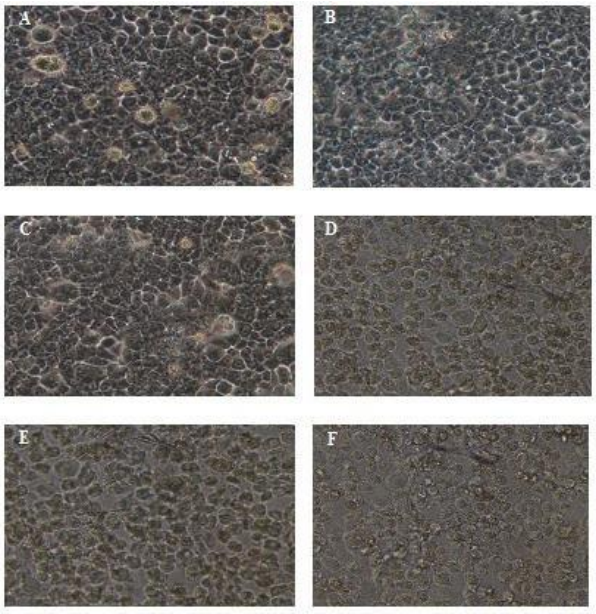

Figure 3: Effect of apigenin on morphological changes in Hep-2 cells

A- untreated Hep-2 cells; B- 25 µg/ml of apigenin; C- 50 µg/ml of apigenin; D- 100 µg/ml of apigenin; E- 200 µg/ml of apigenin; F-30 µg/ml of methotrexate.

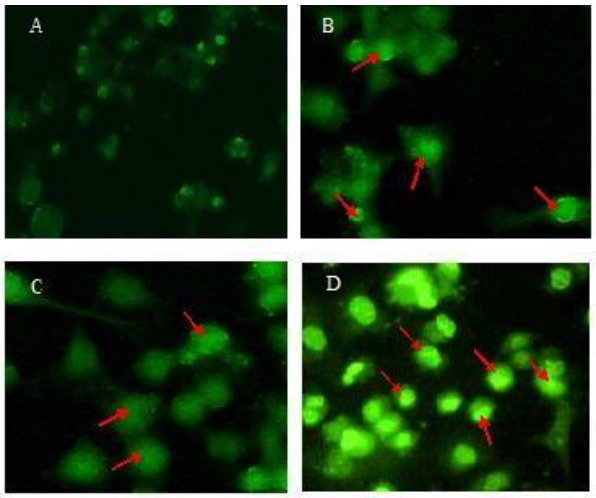

Figure 4: ROS generation in untreated Hep-2 cells and apigenin treated Hep-2 cells

Arrows represent ROS generation in Apigenin treated Hep-2cells. A- untreated Hep-2 cells; B- 25 µg/ml of apigenin; C- 50 µg/ml of apigenin; D- 100 µg/ml of apigenin

DAPI Staining

The apoptotic efficacy of apigenin on Hep-2 cells was assessed using DAPI staining (figure 6). Apigenin at a dose of 50 µg/ml effectively induced cell shrinkage, cell blebbing, cell budding and discrete nuclei fragments in Hep-2 cells.

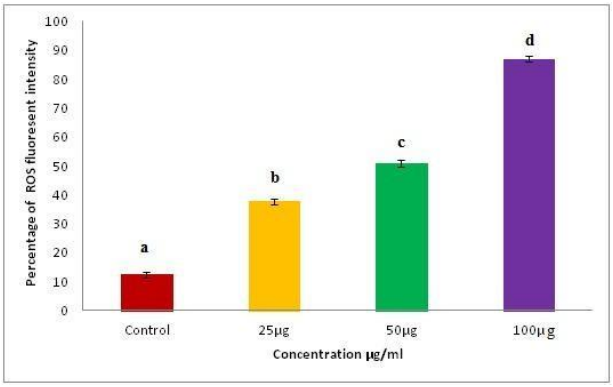

Figure 5: Percentage of ROS fluorescent intensity in untreated Hep-2 cells and apigenin treated Hep-2 cells

Values that do not share a common superscript between the groups differ significantly at $p< 0.05$ (DMRT).

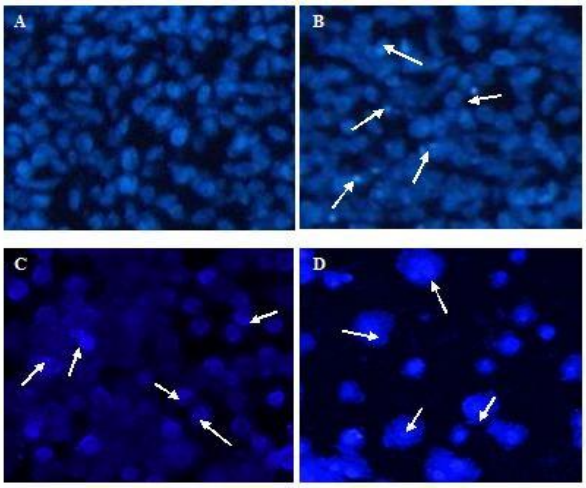

Figure 6: Nuclear damage in untreated Hep-2 cells and apigenin treated Hep-2 cells

Arrows indicate nuclear damage. A- untreated Hep-2 cells; B- 25 µg/ml of apigenin; C- 50 µg/ml of apigenin; D- 100 µg/ml of apigenin

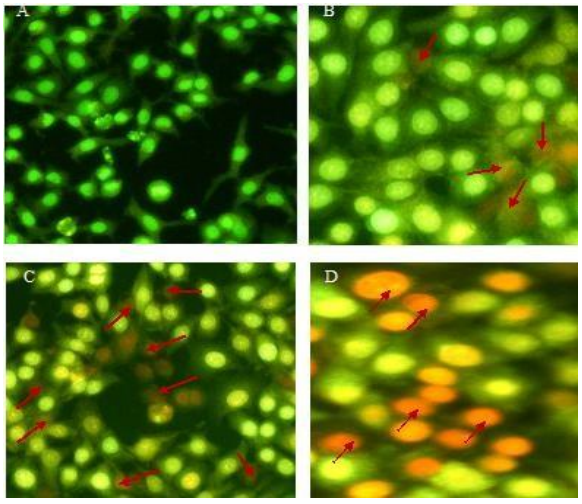

Figure 7: Apoptosis in untreated Hep-2 cells and apigenin treated Hep-2 cells

Arrows indicate apoptosis. A- untreated Hep-2 cells; B- 25 µg/ml of apigenin; C- 50 µg/ml of apigenin; D- 100 µg/ml of apigenin

Apoptotic DNA fragmentation

Figure 8 depicts DNA fragmentation status in untreated and apigenin treated Hep-2 cells. Agarose gel electrophoresis exhibited a number of DNA fragments in apigenin treated Hep-2 cells (50 µg/ml) than the untreated cells.

Expression pattern of apoptotic and invasive markers

Figure 9 and 10 illustrates the expression pattern of apoptotic (p^{53} and Bcl-2) and invasive (MMP-2 and MMP-9) markers using Western blotting and their densitometric analysis respectively in untreated and apigenin treated Hep-2 cells. Apigenin modulated the expression of the above markers in favor of the suppression of Hep-2 cell survival.

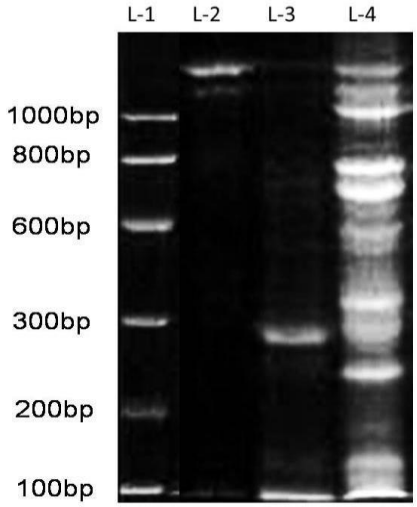

Figure 8: DNA fragmentation in untreated Hep-2 cells and apigenin treated Hep-2 cells

L - 1-100bp DNA ladder
L - 2 DNA Isolated from untreated Hep – 2 cells
L - 3 DNA Isolated from 25 µg/ml of apigenin treated Hep – 2 cells
L- 4 DNA Isolated from 50 µg/ml of apigenin treated Hep – 2 cells.

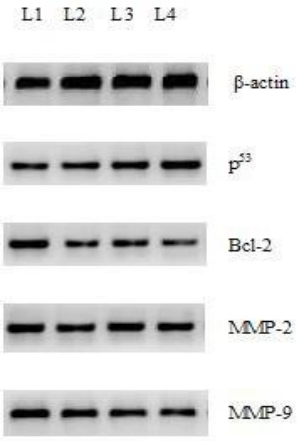

Figure 9: Expression pattern of p53, Bcl-2, MMP-2 and MMP-9 in untreated Hep-2 cells

Lane1: untreated Hep-2 cells; Lane 2: 25 µg/ml of apigenin; Lane 3: 50 µg/ml of apigenin; Lane 4: 100 µg/ml of apigenin

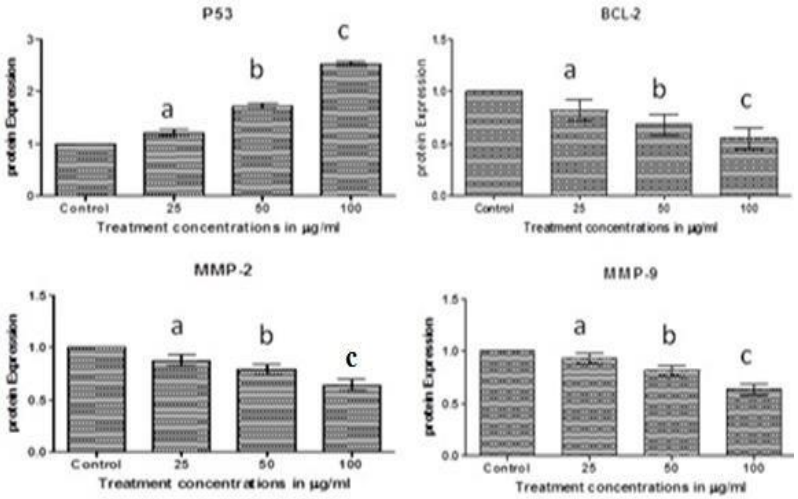

Figure 10: Densitometric analysis of apoptosis related proteins in untreated Hep-2 cells and apigenin treated Hep-2 cells

Values are expressed as mean ± standard deviation (S.D) for three independent experiments. Values that do not share a common superscript between the groups differ significantly at p <0.05 (DMRT)

DISCUSSION

MTT assay, a commonly employed method, is used to assess the cytotoxic effect of natural products and their constituents in Hep-2 cells (Choi & Kim, 2009a). MTT is taken up only by the metabolically viable cells and the resultant purple color developed would indicate the number of viable cells (Sargent, 2003). In the present study, we have assessed the cytotoxic potential of apigenin using a cell viability assay (MTT). Apigenin inhibited the growth of several types of cancer cells. However, the effect of apigenin on Hep-2 cells has not been characterized well. The percentage viability of Hep-2 cells was observed for 24hr after treatment with apigenin at various concentrations (25 µg/ml, 50 µg/ml, 100µg/ml and 200 µg/ml). We, however, observed that apigenin at a concentration of 50 µg/ml exhibited 50 % of cell viability (i.e., IC_{50} was found to be 50µg/ml).We observed maximum reduction in cell viability at a concentration of 200 µg/ml. Apigenin reduced the cell viability in the Hep-2 cells as evidenced by MTT assay, which indicates its anti-cell proliferative potential (cytotoxic effect).

Imbalance in oxidant and antioxidant status leads to a condition known as oxidative stress, which has been implicated in the pathogenesis of various diseases including cancer. Over production of ROS can cause oxidative damage to DNA, proteins and lipids (Cooke, et al*.,* 1997). It has been reported that the rate of cell proliferation is inversely proportional to the generation of reactive oxygen species. Reactive oxygen species also play pivotal role in the induction of apoptosis in cancer cells if they are excessively generated (Kannan & Jain, 2000). Our results suggest that apigenin suppressed the proliferation of Hep-2 cells; probably through reactive oxygen species mediated apoptosis. Oxidants can able to cause necrosis at higher concentrations and apoptosis at lower concentration (Jabs, 1999). Over production of reactive oxygen species has been reported in various cancer cell lines treated with cytotoxic or therapeutic agents. Profound studies have highlighted the role of ROS in apoptosis in various cancer cell lines (Lindeberg & Krogdahl, 1999). We also noticed excess generation of reactive oxygen species in apigenin treated Hep-2 cells. The present study thus suggests that the cytotoxicity was stimulated by apigenin in Hep-2 cells through oxidative stress and, ROS mediator apoptotic mechanism.

Apoptotic nuclear morphology could be assessed with a help of DAPI (4',6-diamidino-2-phenyl-indole) staining technique. Apigenin treated Hep-2 cells were stained with DAPI and the morphological characteristics relevant to apoptosis was analyzed. Apigenin treated Hep-2 cells showed membrane blebbing, cell shrinkage, nuclei fragments and cell budding, which are the characteristic features of apoptosis (Wang, et al., 2007, Choi and Kim, 2009b).

Apoptosis, the programmed cell death, has been recently utilized as an important mechanism to assess the anticancer potential of the test compound (Cheon, et al., 2006). Apoptosis is characterized by morphological events such as nuclear fragmentation, nuclear condensation, cell shrinkage and DNA fragmentation. Apoptosis plays crucial role in the regulation of cell growth, cell development, immune response and clearing the abnormal cells. (Schutte, et al., 1998; Plasier, et al., 1999; Vermes, et al., 1995).

Ethidium bromide and acridine orange dual staining method is widely employed to assess the morphological characteristics of apoptotic cells. While viable cells leave out ethidium bromide, non viable cells allow ethidium bromide into the cells. However, viable cells allow acridine orange, which reacts with DNA to give green nuclear fluorescence. In non-viable cells, ethidium bromide reacts with DNA and yield red orange fluorescence (Grossmann, et al., 1998). Hep-2

cells treated with 25 µg/ml, 50 µg/ml, and 100 µg/ml apigenin for 24hrs showed apoptotic body formation. Based on the above principle, we confirm that apigenin has the potential to stimulate apoptosis in Hep-2 cells, as evidenced by red orange fluorescence in apigenin treated Hep-2 cells. In the present study, Hep-2 cells treated with 25 µg/ml, 50 µg/ml and 100 µg/ml apigenin for 24 hrs showed apoptotic body formation.

Agarose gel electrophoresis is used to find out the DNA fragmentation in cancer cell lines before and after treatment with a test compound (Collins, et al., 1992; Cohen, et al., 1992). In the present study, we noticed a number of DNA fragments in apigenin treated Hep-2 cells, which clearly indicates the apoptotic potential of apigenin.

p^{53}, the guardian of the genome, has a vast potential in the treatment of certain tumors (Shao, et al., 2010 and Suzuki & Matsubara, 2011). P53 can protect normal cell growth and can induce malignant cell death (Levine & Oren, 2009). P53 has a multiple functions including a role in cell cycle arrest, DNA damage repair and initiation of apoptosis (Meek, 2009**;** Junttila & Evan, 2009). Bcl-2, the antiapoptotic protein, is negatively regulated by p^{53}. It has been suggested that higher or lowered expression of Bcl-2 family proteins in the Bcl-2 family is tightly linked to carcinogenesis. Over expression of Bcl-2 ensures the survival advantage in the tumor cells and thereby promotes tumorigenesis (Liu, et al., 1996). Bcl-2, an antiapoptotic protein, inhibits apoptosis by preventing the redistribution of cytochrome-c in response to multiple death inducing signals **(**Vander Heiden & Thompson*,* 1999; Borner, 2003).

To evaluate whether apigenin stimulated the apoptotic pathway in Hep-2 cells, we analyzed the expression pattern of the apoptotic markers p^{53} and Bcl-2 using Western Blotting (Haffty & Glazer, 2003; Kumar et al., 2008). Chan, et al., (2012) reported that apigenin induces apoptosis via tumor necrosis factor receptor and Bcl-2 mediated pathway and enhances susceptibility of head and neck squamous cell carcinoma to 5-fluorouracil and cisplatin. Lu, et al., (2011) reported that ROS played critical role in apigenin-induced apoptosis. Our results also showed that the expression of p^{53} (proapoptotic) was upregulated and Bcl-2 (antiapoptotic) expression was downregulated in apigenin treated Hep-2 cells, which indicate that apigenin stimulated the Hep-2 cell death through apoptotic mechanism (Gamet-Payrastre, et al., 2000).

Matrix metalloproteinases, the family of zinc dependent endopeptidases, are responsible for the degradation of extracellular matrix components (Malemud, 2006; Ohbayashi, 2002). Among 22 members of human MMP gene family, MMP-2 and MMP-9 are linked with the invasion and metastasis of malignant cells (Miyamori, et al., 2001**;** Ohnishi, et al., 2002). MMP-2 and MMP-9 expressions are increased in several cancers and thus researchers are utilizing MMP-2 and MMP-9 as a preferential target in cancer therapy (Thant, et al., 2000). In the present study, Western blotting results showed that the expression of MMP-2 and MMP-9 are downregulated in apigenin treated Hep-2 cells. Apigenin mediated anticancer effect by stimulating gap functional and intracellular communication, inhibition of mutagenesis, angiogenesis and tumorigenesis (Dobrydneva, et al., 1999). The results of the present study also indicate that apigenin has the potential to inhibit the process of angiogenesis by downregulating MMP-2 and MMP-9 in Hep-2 cells. The present study thus suggests that apigenin inhibited the cell proliferation of Hep-2 cells (cytotoxic potential) through its proapoptotic and anti-angiogenic potential.

The overall findings of the present study suggest that apigenin inhibited the cell proliferation (MTT assay) in Hep-2 cells, by inducing apoptosis (dual staining EB/AO), modulating the expression pattern of p^{53} and Bcl-2 (Western blotting), stimulating the excessive generation of ROS (DCFH-DA method), by causing DNA fragmentation (Agarose gel electrophoresis), nuclear damage (DAPI staining), and by inhibiting angiogenesis (MMP-2 and MMP-9 (Western blotting)).

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