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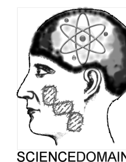

# Ferulic Acid Reduces Cell Viability through Its Apoptotic Efficacy: An *In vitro* Approach

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author SM designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author RS managed the literature searches. Authors MMP and TR performed the in vitro analysis. Author VIHI managed the experimental process. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** Ferulic acid, a well known dietary phenolic antioxidant, possesses diverse pharmacological and biochemical effects, including anti-inflammatory, hepatoprotective, antidiabetic and anticancer properties. The present study explores the cytotoxic potential of ferulic acid using Hep-2 cell line by analyzing its effect on cell viability, reactive oxygen species generation, apoptotic induction, nuclear damage, DNA fragmentation and expression of apoptosis related proteins.

**Materials and Methods:** The effect of ferulic acid (2.5, 5, 10, 20 and 40 µg/ml) on Hep-2 cells viability for 24 hr was determined by MTT assay. To substantiate the cytotoxic effect of ferulic acid, the intracellular ROS level was determined using DCFH-DA assay; apoptosis by dual staining; nuclear damage by DAPI staining; DNA fragmentation by using agarose gel electrophoresis; apoptosis related proteins by western blotting.

**Results:** Ferulic acid significantly inhibited the Hep-2 cell growth in a dose dependent manner and ferulic acid treated Hep-2 cells exhibited features of apoptosis and increase in nuclear damage and DNA fragmentation. We also observed excess reactive oxygen species generation in ferulic acid

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treated Hep-2 cells. Apoptosis related proteins (p53, Bcl-2, Bax, Caspase 3 & Caspase 9) were significantly modulated in favour of programmed cell death in ferulic acid treated cells.
**Conclusion:** We thus conclude that the cytotoxic potential of ferulic acid might be due to its role in apoptosis induction, excessive ROS generation and DNA fragmentation in Hep-2 cells.

*Keywords: Apoptosis; ferulic acid; Hep-2 cell; DNA fragmentation; oxidative stress.*

## 1. INTRODUCTION

Carcinogenesis arises due to disturbances in the existing balance between the rate of cell proliferation and cell death. Cell shrinkage, nuclear fragmentation and apoptotic body formation are considered as primary indicators of apoptosis [1,2]. Dysregulation in cell proliferation and apoptosis are the hallmark of cancer development [3,4]. Extensive studies pointed out that phytochemicals suppress or inhibit the carcinogenesis through modulation of apoptotic and cell proliferative marker's expression pattern [5,6]. Ferulic acid is one such phytoconstituent exhibited diverse pharmacological effects including anticancer and pro-apoptotic properties [7]. Ferulic acid, a well known phenolic antioxidant, is abundantly present in rice bran, green tea, fruits, vegetables and coffee beans [8]. Ferulic acid inhibited the viability of various cancer cell lines [9,10]. Ferulic acid has the vast potential to scavenge wide range of reactive oxygen species including superoxide and hydroxyl radicals [11]. Ferulic acid suppressed the formation of tumors in various experimental carcinogenesis including DMBA-induced oral carcinogenesis [12]. Previous studies from our laboratory explored the anticancer potential of ferulic acid in DMBA-induced skin, mammary and oral carcinogenesis [13-15]. Diverse pharmacological, biochemical and therapeutic effects of ferulic acid are well documented. The present study was designed to investigate the cytotoxic and apoptotic potential of ferulic acid in Hep-2 cell lines by assessing the cell viability, ROS levels, apoptotic induction, nuclear damage and DNA fragmentation.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Ferulic acid was purchased from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. Fetal Bovine Serum (FBS) was purchased from Sigma USA. Dulbecco's Modified Eagle's Medium (DMEM), Streptomycin, Penicillin-G, Phosphate Buffered Saline (PBS), 3-4,5 dimethylthiozol-2-yl-2,5-diphenyltetrazoliumbromide (MTT), 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), Acridine orange (AO), Ethidium bromide (EB), 4', 6-diamidino-2-phenylindole (DAPI), Ethylene diamine tetra acetic acid (EDTA), Triton X-100, Phenol, Chloroform, Ethanol, Sodium acetate, Dimethyl sulfoxide (DMSO), Acrylamide, Hydrochloric acid, Ammonium persulphate, Tetramethylene diamine (TEMED), Glycine, Bromophenol blue, Glycerol, Tris Buffer Saline, Tween 20, 2-Mercapto ethanol and Sodium chloride were purchased from Himedia Laboratories, Mumbai. p53, Bcl-2, Bax, Caspase 3 and Caspase 9 primary antibodies were purchased from Abcam, Merk Germany C.A. Alkaline phosphatase- conjugated secondary antibody was purchased from Himedia Laboratories, Mumbai.

### 2.2 Hep-2 Cell Line and Its Maintenance

Hep-2 cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India and was cultured in Dulbecco's Modified Eagle's Medium (DMEM). The cell line was maintained in cell culture laboratory, Pondicherry Centre for Biological Science, Pondicherry. This medium was 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 37ºC and the experiments were carried out after confluency stage was attained. The medium was replaced every 2 days and the maintenance was strictly followed in accordance with the standard methods. The cells were dissociated with trypsin phosphate versene glucose in phosphate buffered saline.

### 2.3 Drug Exposure

Hep-2 cells from log phase cultures were incubated with various concentrations of ferulic acid (2.5 – 40 µg/ml) at 37ºC in a $CO_2$ incubator for 24 hr. Ferulic acid was dissolved in DMSO and the final concentration of DMSO in media was less than 0.1%.

### 2.4 MTT Assay

The effects of ferulic acid on cell viability of Hep-2 cells are assessed by the method of Mosmann

[16]. MTT assay measures the reduction of yellow tetrazolium salt (MTT) by the mitochondrial dehydrogenase of metabolically active cells to form insoluble dark purple colored formazan product. The amount of formazan product is directly proportional to the number of living cells present in the culture. The results are expressed at the percentage of viable cells with respect to the control. The medium effective dose ($IC_{50}$), which is the amount of samples able to inhibit cell viability by 50 % and was calculated graphically.

## 2.5 Measurement of Intracellular ROS

The intracellular ROS level was determined according to the method of Pereira et al. [17] using a non fluorescent probe 2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA). This non fluorescent probe can diffuse into the intracellular matrix of cells, where it is acted upon by intracellular esterase to form polar 2’, 7’-dichlorodihydrofluorescein (DCFH), which gets trapped inside the cells and oxidised by intracellular oxidants to a highly fluorescent 2’, 7’-Dichlorofluorescein (DCF).

## 2.6 DAPI Staining

The extend of apoptosis in drug treated Hep-2 cells was measured using DAPI staining [18]. In this method, the harvested Hep-2 cells were treated with ferulic acid for 24 hr. After incubation, the cells were washed with PBS and fixed with ice-cold TCA followed by washing with 70%, 80%, 90% and absolute ethanol. The cells were then permeabilized with Triton-X and stained with DAPI for 3 minutes. The stained cells were coverslipped with 90% glycerol and observed under a fluorescent microscope.

## 2.7 Measurement of Apoptotic Induction Using Acridine Orange/Ethidium Bromide Dual Staining

The fluorescence morphological analysis of cell death was carried out according to the method of Baskic et al. [19]. Hep-2 cells were seeded at $5x10^4$ cells /well in a 6 well plate and incubated for 24 hr. After incubation the cells were treated with ferulic acid for 24 hr. At the end of the treatment the cells were collected and washed three times with PBS. The plate were then stained with acridine orange and ethidium Bromide in the ratio of 1:1 (100 µg/ml) for 5 min and examined immediately under fluorescent microscope 200x magnification. While acridine orange is taken up by both viable and non- viable cells, ethidium bromide is taken up only by non viable cells. While acridine orange emits green fluorescent, ethidium bromide emits red fluorescent if intercalated into DNA. The cells were categorised into four types as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei). A total 300 cells were counted in each sample.

## 2.8 Apoptotic DNA Fragmentation

This method involves the isolation of apoptotic DNA fragment by lysing the isolated nuclei of apoptotic cells with NP-40 buffer. The apoptotic DNA fragments were released into the supernatant was separated by 1% agarose gel electrophoresis. The isolation of apoptotic DNA fragment was carried out according to the method of Herrmann et al. [20]. The harvested Hep-2 cells were pelleted by centrifugation and the pelleted cells were then treated for 10 seconds with lysis buffer. After lysis, the centrifugation was carried out for 5 minutes at 16000 g. The supernatant was collected and the extraction is repeated with same amount of lysis buffers. The collected supernatants were mixed with 1% SDS and treated with RNase A at 56ºC, followed by digestion with proteinase K for 2 hr at 37ºC. The DNA was then precipitated with ethanol immediately after treating with ammonium acetate. The precipitate was dissolved in gel loading buffer and separated by electrophoresis in 1% agarose gels.

## 2.9 Western Blotting

Western blotting is a commonly employed technique to detect and characterize specific proteins in a tissue sample. The technique is based on the ability of protein antigens in the tissue samples to bind to their specific antibodies. The protein concentration was determined in the supernatant collected from the lysate according to the method of Bradford et al. [21]. The cell lysate containing 50 µg protein was boiled for at 95ºC in SDS sample buffer and loaded on to a 12% SDS polyacrylamide gel. After performing electrophoresis the protein bands (blot) were transferred to PVDF membrane for 1½ hr with a solution containing 5% non- fat dry milk, 10 mmol tris-HCl (8.0 pH), 150 mmol sodium chloride and 0.1% Tween 20

(TBST). The PVDF membrane containing the blot was incubated with primary antibodies polyclonal rabbit / anti-rabbit Ig; p53, Bcl-2, Bax, Caspase 3 and Caspase 9 at 4ºC over night. After incubation the membrane washed with TBST at regular interval of 6 min for 30 min in TBST. Then the blots were incubated with alkaline phosphatase-conjugated secondary antibody for 1 hr at room temperature. The protein bands were then developed using NBT/BCIP solution and the results were analysed densitometrically using Image J software.

### 2.10 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.

## 3. RESULTS AND DISCUSSION

### 3.1 MTT Assay

The effect of ferulic acid on cell viability by MTT assay and morphological changes in Hep-2 cells for 24 hr are depicted in Fig 1A and Fig. 1B (A-D). The Hep-2 cells were incubated with different concentrations of ferulic acid (2.5, 5, 10, 20 and 40 µg/ml) for 24 hr. Ferulic acid inhibited the viability of Hep-2 cells in a dose dependent manner and 30%, 50% and 90% inhibitory concentrations ($IC_{30}$, $IC_{50}$ and $IC_{90}$) for ferulic acid was attained at 4.85 µg/ml, 13.2 µg/ml and 37.5 µg/ml for 24 hr using MTT assay.

MTT assay is commonly employed to assess the cytotoxic potential of the test compound based on the viability of test (cancer) cells. Metabolically active viable cells can cleave MTT into a purple product which can be measured colorimetrically [22]. The intensity of the color is used to assess the cytotoxic efficacy of the test compound. Ferulic acid at a concentration of 2.5 to 40 µg/ml for 24 hr significantly decreased the viability of Hep-2 cells in a dose dependent manner as evidenced by MTT assay, which shows its cytotoxic potential.

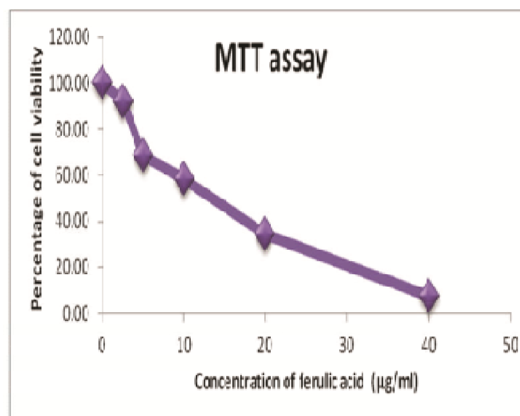


**Fig. 1A. The effect of ferulic acid on cell viability for 24 hr using MTT assay**

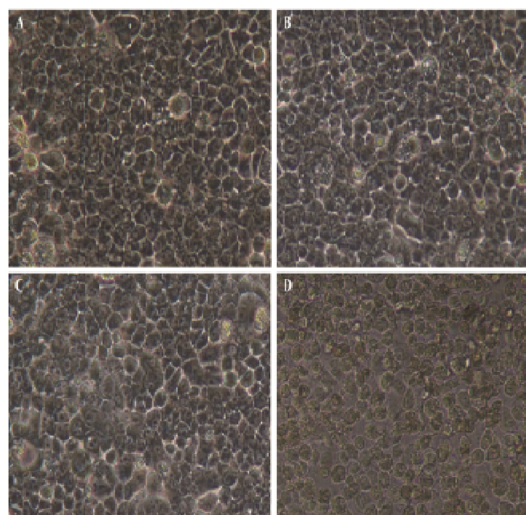


**Fig. 1B. Morphological changes in control and ferulic acid treated Hep-2 cells for 24 hr**
(Photographs magnification: 20X)
*A-Control untreated HEp-2 cells; B- $IC_{30}$ of ferulic acid (4.85 µg/ml); C- $IC_{50}$ of ferulic acid (13.2 µg/ml); D- $IC_{90}$ of ferulic acid (37.5 µg/ml)*

### 3.2 Measurement of Intracellular ROS

Fig. 2 (A-D) reveals the effect of ferulic acid on the status of ROS accumulation in Hep-2 cells for 24 hr using DCFH-DA staining. Ferulic acid at concentrations of 4.85 µg/ml, 13.2 µg/ml and 37.5 µg/ml significantly enhanced the generation of ROS as compared to control Hep-2 cells. Under fluorescence microscope, ferulic acid treated Hep-2 cells revealed bright green fluorescence than the untreared Hep-2 cells. The fluorescence intensity was then measured spectrofluorimetrically (Fig. 3).

ROS affect normal cellular signaling as well as gene regulation if they are excessively generated [23]. Intracellular ROS plays pivotal role in apoptotic induction by causing DNA fragmentation and nuclear damage [24]. Over production of ROS has been implicated in oxidative stress, loss of cell function and apoptosis [25,26]. Profound literature documented the role of reactive oxygen species in the induction of apoptosis [27,28]. DCFH-DA fluorescence assay could help to investigate the ROS generating potential of the cytotoxic compounds. Ferulic acid has been suggested as a prooxidant in various *in vitro* studies on cancer [10,29]. In the present study, ferulic acid treated Hep-2 cells exhibited green fluorescence which proves the excess generation of ROS. Excessive generation of ROS in the ferulic acid treated Hep-2 cells suggest that ferulic acid mediated apoptosis through excessive generation of ROS.

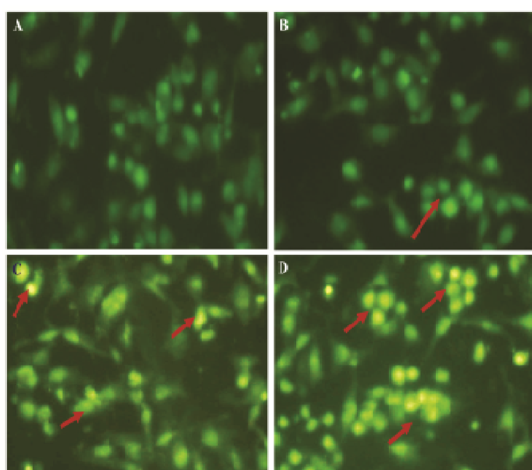


**Fig. 2. ROS accumulation in control and ferulic acid treated Hep-2 cells for 24 hr**

*Red arrow represents an excessive ROS generation in ferulic acid treated Hep-2cells*

*A-Control untreated HEp-2 cells; B- $IC_{30}$ of ferulic acid (4.85 µg/ml); C- $IC_{50}$ of ferulic acid (13.2 µg/ml); D- $IC_{90}$ of ferulic acid (37.5 µg/ml)*

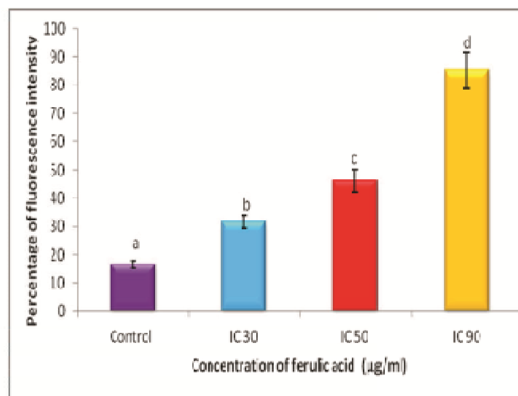


**Fig. 3. Percentage of ROS fluorescence intensity in control and ferulic acid treated Hep-2 cells**

*A-Control untreated HEp-2 cells; B- $IC_{30}$ of ferulic acid (4.85 µg/ml); C- $IC_{50}$ of ferulic acid (13.2 µg/ml); D- $IC_{90}$ of ferulic acid (37.5 µg/ml)*

*Values are expressed as Mean ± Standard deviation (S.D) for three independent experiments. Values that do not share a common superscript (a,b,c and d) between the groups differ significantly at $p < 0.05$ (DMRT)*

## 3.3 DAPI Staining

In order to investigate the apoptotic potential of ferulic acid, morphological analysis using DAPI staining was carried out in Hep-2 cell lines. Ferulic acid at doses of 4.85, 13.2 and 37.5 µg/ml for 24 hr exhibited prominent apoptotic potential as evidenced by cell shrinkage, cell budding and discrete nuclei fragments (Fig. 4 (A-D)).

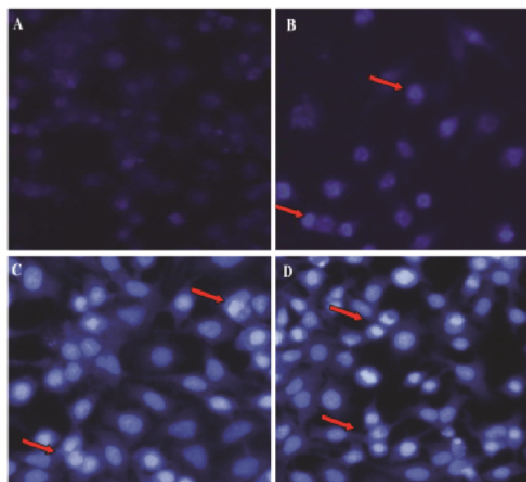


**Fig. 4. Nuclear damage in control and ferulic acid treated Hep-2 cells using DAPI**

*Red arrow indicates nuclear damage in the Hep-2 cells treated with ferulic acid*

*A-Control untreated HEp-2 cells; B- $IC_{30}$ of ferulic acid (4.85 µg/ml); C- $IC_{50}$ of ferulic acid (13.2 µg/ml); D- $IC_{90}$ of ferulic acid (37.5 µg/ml)*

In cancer cells the apoptotic induction by cytotoxic agents is characterized by morphological changes and biochemical events [30]. DAPI staining is an attractive procedure to evaluate the nuclear damage occurring in apoptosis. Nuclear damage involves nuclear shrinkage, nuclear membrane disruption, DNA

damage and chromatin condensation. DAPI intercalates with A-T rich base pairs of the minor groove in the DNA and emits blue fluorescence [31]. Apoptotic cells exhibit blue colored fluorescence, when they are stained with DAPI. In the present study, we observed blue colour fluorescence in the ferulic acid treated Hep-2 cells, which indicates the apoptotic potential of ferulic acid. Though the mechanism is unclear, ferulic acid might have enhanced the intercalation of DAPI with the base pairs of DNA.

### 3.4 Measurement of Apoptotic Induction Using Acridine Orange/Ethidium Bromide Dual Staining

The apoptotic characterization was analysed using AO/EB dual staining. Ferulic acid treated Hep-2 cells and control Hep-2 cells are depicted in Fig. 5 (A-D). AO/EB staining differentiates the cells as follows; homogenous bright green nuclei (viable cells), early apoptotic cells (green nuclei), bright green patches or fragments (orange to red nuclei with condensed chromatin) and necrotic cells (homogenous orange to red nuclei with integral structure). Hep-2 cells treated with ferulic acid for 24 hr exhibited early apoptotic cells (4.85 µg/ml; green nuclei), late apoptotic cells (13.2 µg/ml; orange nuclei) and necrotic cells (37.5 µg/ml; red nuclei). The cell death due to apoptotic process is significantly observed in Hep-2 cells treated with ferulic acid for 24 hr than the untreated Hep-2 cells.

Apoptosis is a programmed cell death, in which the unwanted cells are removed from the body without releasing harmful substances into the surrounding area. Any defect in the regulation of apoptosis pathway leads to several disorders, including cancer [32]. Apoptotic induction in cancer cells is now recognized as a major target to investigate the antitumor therapeutic agents from natural products [33]. Ethidium bromide/acridine orange dual staining is commonly employed to assess the apoptotic induction potential of cytotoxic agents [34]. Present results revealed that ferulic acid treated Hep-2 cells exhibited red-orange fluorescence, which indicated the apoptotic potential of ferulic acid. Furthermore ferulic acid treated cells showed DNA condensation and fragmentation, which confirm the apoptotic induction in these cells.

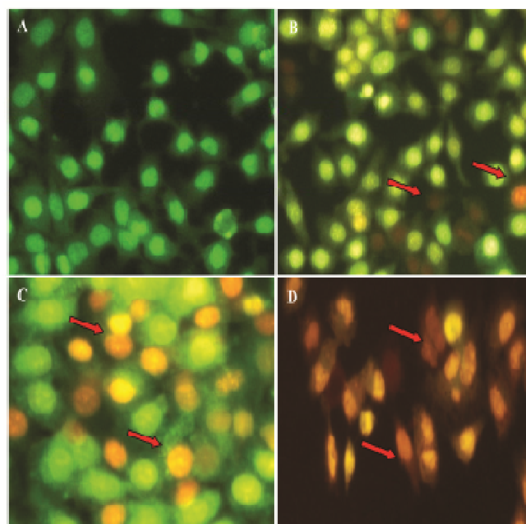


**Fig. 5. Apoptosis in control and ferulic acid treated Hep-2 cells using acridine orange and ethidium bromide**

*Red arrow indicates early apoptotic cells (Fig. 5B), late apoptotic cells (Fig. 5C) and necrotic cells (Fig. 5D), A-Control untreated HEp-2 cells; B- $IC_{30}$ of ferulic acid (4.85 µg/ml); C- $IC_{50}$ of ferulic acid (13.2 µg/ml); D- $IC_{90}$ of ferulic acid (37.5 µg/ml)*

### 3.5 Apoptotic DNA Fragmentation

DNA fragmentation in control and ferulic acid treated Hep-2 cells by their DNA ladder are shown in Fig. 6. We noticed significant increase in DNA fragmentation in Hep-2 cells treated with ferulic acid (4.85 µg/ml, 13.2 µg/ml) for 24 hr. DNA fragmentation assay using agarose gel electrophoresis could also help to evaluate the apoptotic potential of the test compound. Agarose gel electrophoresis revealed a typical DNA ladder for ferulic acid treated Hep-2 cells which showed more DNA fragmentation, which further proves the cytotoxic or apoptotic potential of ferulic acid.

### 3.6 Western Blotting

The expression of apoptosis related proteins (p53, Bcl-2, Bax, Caspase 3 and 9) and their densitometric analysis in control and ferulic acid treated Hep-2 cells are shown in Fig. 7 and Fig. 8. We observed overexpression of p53, Bax, Caspase 3 & 9 and decreased expression of Bcl-2 in ferulic acid treated Hep-2 cells as compared to untreated Hep-2 cells.

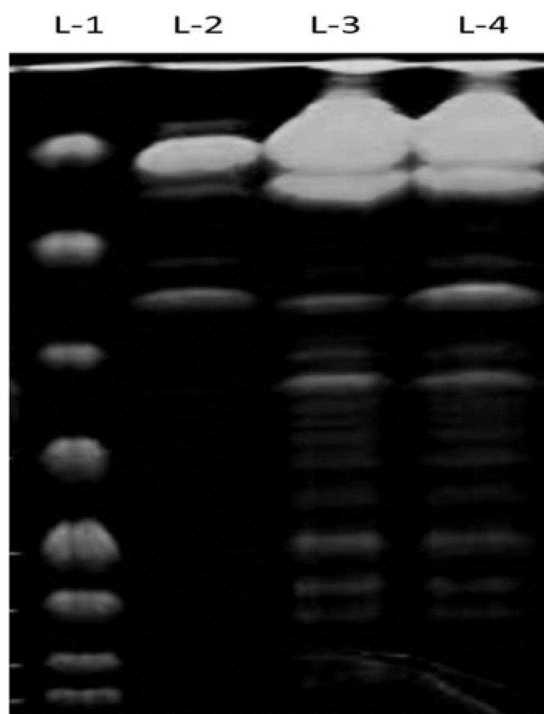


**Fig. 6 DNA fragmentation in control and ferulic acid treated Hep-2 cells**

*Lane 1: 1 Kb DNA Marker, Lane 2: DNA isolated from untreated control HEp-2 cells, Lane 3: DNA isolated from $IC_{30}$ of ferulic acid (4.85 µg/ml) treated HEp-2 cells, Lane 4: DNA isolated from $IC_{50}$ of ferulic acid (13.2 µg/ml) treated HEp-2 cells*

Imbalance in proapoptotic and antiapoptotic mechanism leads to lack of cell death, which is the characteristic feature of carcinogenesis. p53, the tumor suppressor gene, plays a prominent role in various biological processes including cell cycle arrest and apoptosis. Normally, p53 does not accumulate in normal cells due its rapid intracellular turnover [35]. p53 triggers cytochrome C release by permeablizing the outer mitochondrial membrane and by forming complexes with Bcl-2 proteins [36]. Bax, apoptosis regulator, is the first identified proapoptotic member of the Bcl-2 gene family. Bax is involved in p53-mediated apoptosis and its expression is upregulated by p53 [37]. Over expression of Bcl-2, an important antiapototic protein, has been identified in several cancers, including breast, oral and prostate cancer [38-40]. We noticed an increase in Bax to Bcl-2 ratio in the ferulic acid treated Hep-2 cells, which implies that ferulic acid triggered cell death via upregulation of Bax (pro-apoptotic) and downregulation of Bcl-2 (anti-apoptotic). Hou et al. [41] reported that ferulic acid inhibited the proliferation of ECV 304 cells through increasing the production of nitric oxide (NO), which subsequently downregulated the ERK1/2 pathway.

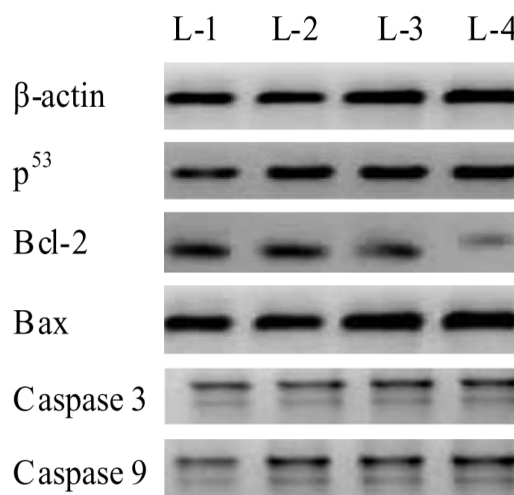


**Fig. 7. The expression of apoptosis related proteins (p53, Bcl-2, Bax, Caspase 3 and 9) in control and ferulic acid treated Hep-2 cells**

*Expression pattern of p53, Bcl-2, Bax, Caspase 3 and Caspase 9 by western blotting has been represented here. β-actin served as internal control. Lane 1: Control untreated; Lane 2: $IC_{30}$ of ferulic acid (4.85 µg/ml); Lane 3: $IC_{50}$ of ferulic acid (13.2 µg/ml); Lane 4: $IC_{90}$ of ferulic acid (37.5 µg/ml)*

It has been reported that phytochemicals can stimulate apoptosis in cancer cells through activation of caspase-3, 8 and 9 [42,43]. Caspases, particularly caspase 3 and 9, are involved in the execution of apoptosis [44]. Activation of caspase 3 in turn results in PARP cleavage which then degrades DNA into nucleosomal fragments [45]. Our study corroborates these observations. Over expression of p53, Bax and caspase 3 and 9 and decreased expression of Bcl-2 in ferulic acid treated Hep-2 cells confirm the process of apoptosis as well as the cytotoxic efficacy of ferulic acid.

## 4. CONCLUSION

The present study thus utilizes MTT assay, DCFH-DA staining, DAPI staining, Dual staining (AO/EB) and DNA fragmentation assay to explore the cytotoxic potential of ferulic acid in Hep-2 cells. Based on our findings, we conclude that the cytotoxic potential of ferulic acid on Hep-2 cells relies on its apoptotic induction potential.

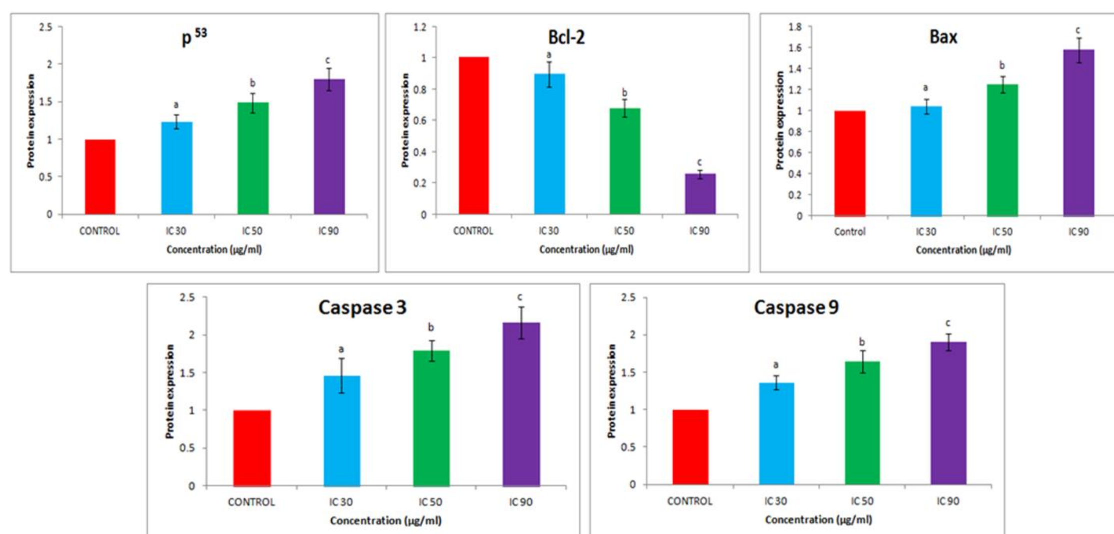


**Fig. 8. Densitometric analysis of apoptosis related proteins p53, Bcl-2, Bax, Caspase 3 and Caspase 9 in control and ferulic acid treated Hep-2 cells**

*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)*

## CONSENT

Not applicable.

## ETHICAL APPROVAL

Not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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