

*Full Length Research Paper*

# Molecular and biochemical evaluation of anti-proliferative effect of (*Cichorium endivia*, L.) phenolic extracts on breast cancer cell line: MCF7

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Medicinal plants are considered to be the most hopeful way for cancer treatment. The *Cichorium endivia*, L. plant materials were collected from Tanuma, Saudi Arabia. Methanol extraction for the phenolic compounds was carried out and the HPLC analysis showed that, the extract containing four main compounds with different concentrations. The anticancer activity of the root extract was examined on breast cancer cell line MCF7 compared with the anticancer 5 FU (5-fluorouracil). Cytotoxicity of the root extract against the MCF7 line was 401ug/mL but it was 0.67 ug/mL with the 5 FU. The gene expression for the DNA cancer markers; P53, Bcl2, TNF and interleukin IL-4, IL-6 and IL-2 were examined using real time PCR. The expression of the P53 and TNF was high both in cells treated with FU and root extract. Expression of Bcl2 was high in the cell line treated with root extract compared with the FU, yet this expression still was low compared with the control ones. The expression level of IL-2, IL-4 decreased in the examined cell lines treated with both root extract and with 5FU as well. In case of the IL-6 expression was high in cells treated with the extract compared with the treated cells with 5FU and control cell line. Thus, *Cichorium endivia*, which contains a combination of phenolic compounds, represents an enjoyable means of anticancer especially for breast cancer.

**Key words:** Anti-proliferative, *Cichorium endivia*, Phenolic extracts, Breast Cancer and Cell lineMCF7.

## INTRODUCTION

Herbal medicine has become a subject with global importance, making an impact on both world health and international trade. Medicinal plants continue to play an innermost role in the healthcare system of large proportions of the world's population Akerele, (1988). This is mainly true in developing countries, where herbal medicine has a long and uninterrupted history of use. Moreover, the economic benefits of the medicinal plants increase in both developing and industrialized nations WHO (1998). It was clear that the continuous usage of herbal medicine by a large proportion of the population in the developing countries is largely due to the high cost of western pharmaceuticals and healthcare. In addition, herbal medicines are more acceptable in these countries from their cultural and spiritual points of view

Cunningham, (1988). The usage of plants for medicinal remedies is an integral part of the South African cultural life, and this is unlikely to change in the years. Actually, it is estimated that 27 million South Africans use herbal medicines from more than 1020 plant and 150 animal species (Williams, 1996 and Mander, (1997).

*Cichorium sp.* a member of family Asteraceae has been traditionally used as liver protectant and claimed as renal protective and anti-inflammatory. It contains many essential lipids, vitamins and a variety of sugars (Hasan et al., 1990). Enk et al., (2004) had tested the ethanolic extracts of roots, stalks, and inflorescences of populations of wild *Cichorium endivia* subsp. *divaricatum* in terms of protection against sunburn, and in prevention of UVB-induced pyrimidine dimer formation and IL-6 mRNA expression in the human keratinocyte cell line (HaCaT) as well. They proved that application of the *Cichorium endivia* root extract on the skin prior to UVB irradiation totally prevented erythema.

Hasan et al., (1990) reported that to evaluate the plant's methanolic extract for *in vitro* cell growth supporting activity on rat hepatocytes primary culture, the plant extract was evaluated at five different concentrations. At highest concentration of plant extract, cell viability was found to be 66.83%, whereas, at lowest concentrations, cell viability was 24.12%. Other studies showed that the different fractions of alcoholic extract and one phenolic compound AB-IV of seeds of *Cichorium intybus* Linn were screened for antihepatotoxic activity on carbon tetrachloride (CCl<sub>4</sub>)-induced liver damage in albino rats (Ahmed et al., 2003). The degree of protection was measured using biochemical parameters like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALKP), and total protein (TP). The methanol fraction and the anti-hepatic compound (AB-IV) were found to possess a potent antihepatotoxic activity comparable to the standard drug Silymarin (Silybon-70) (Ahmed et al., 2003). The aim of this work is to examine the anticancer activity of the phenolic extract of weed plant *Cichorium endivia*. Three different cell lines were examined to discover which type of cancer should be treated by these compounds.

## MATERIALS AND METHODS

### Plant materials

The *Cichorium endivia*, L. family Asteraceae, plant materials were collected from different regions in Tanuma, Saudi Arabia. Shoot and root parts were separated and washed with distilled water, dried on tissue paper and then grind using blender.

### Extraction of total phenolics content from plant roots

Total polyphenolic compounds in the plant root were extracted by methanol according to Anokwuru et al., (2011). The cichorium roots were collected washed very well with sterile distilled water, dried and ground to powder to obtained fine particles. Twenty gram of the root powder was soaked with 250mL of methanol for 72h. The sample filtrate was concentrated using the rotary evaporator at 40C. The dried extract were weighed and stored in frozen.

### HPLC analysis

A 100 mg of the dried methanolic extracted polphenolic compounds was subjected to the HPCL analysis according to Dittmann et al., (2004). The HPLC equipment consisted of a HP 1100 system (Hewlett–Packard, Waldbronn) with high pressure mixing pump, solvent degasser, column thermostat, photodiode array detector, autosampler and HP Chemstation. Separations were performed on a LiChrospher 100 RP-18e cartridge (5 ml particle size, 125 mm x 4 mm i.d.; Merck, Darmstadt). Gradient elution was carried out with acetonitrile (solvent A) and 10 mM aqueous ammonium acetate (solvent B1, angelica extract), or 25 mM aqueous ammonium acetate (solvent B2, chamomile extract). The separation of latex extract was achieved with the

following gradient profile: 6.964 Catechin (2.132 min) to 1.659 sinnapic acid (20 min). The flow rate was 1 ml/min, and detection was carried out at 230 nm. The injection volume was 10 µl from the crude extract.

### Breast cancer cell line

Breast cancer cell line (MCF7), derived from human, were obtained from the American Type Tissue Collection (ATCC). They were cultured in minimal Eagle's medium (MEM) supplemented with 100 ml/L fetal bovine serum (FBS), 2 mM/L glutamine, 1.5 g/L sodium bicarbonate, 1.0 mM/L nonessential aminoacids, 1.0 mmol/L sodium pyruvate, and incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Cytotoxicity assay (MTT assay)

The cytotoxicity assay was carried out as previously described by [11]. Briefly, between 3000 to 6000 cells, depending on the growth characteristics were seeded in each well of a 96 well microtiter plate, and incubated for 24 h at 37 °C. Different concentrations of the plant root extract 50, 100, 250 and 500 µg (in quadruplicates) were added to the exponentially growing cells. Cell controls in absence of the compound were included with different concentrations of 5FU (1, 2.5, 5 and 10 µg). After an incubation period of 24 h at 37 °C, the MTT assay was performed following the manufacturer's instructions. The absorbance values at 570 nm were recorded in an ELISA plate reader (Meterech 960). The percentage of living cells was calculated according to the following equations:

$$\% \text{ of living cell} = \frac{\text{Absorbance of sample} - \text{Absorbance of DMSO(Blank)}}{\text{Absorbance of control} - \text{Absorbance of DMSO(Blank)}} \times 100$$

The IC<sub>50</sub> for both cell lines were determined by linear regression from dose-response curves.

### Real time PCR and gene expression of cancer DNA markers (p53, Bcl2 and TNF) and immune response markers (IL-4, IL-6 and IL-2) in the treated and non treated cell lines 1- Extraction of total RNA from treated cell lines for 6 hours

The best concentrations of the plant root extract which gave a high toxicity with examined breast cancer cell line in separate manner were used to treat the cell lines separately for 6 hours with the same previous conditions. The treated cell lines were collected separately and subjected to RNA extraction. About 10<sup>6</sup> cells were subjected to RNA extraction using the RNA extraction Mini Kit according to manufacturer's instructions (QIAGEN, Germany). The resultant RNA was dissolved in DEPC-treated water, quantitated spectrophotometrically and analyzed on 1.2% agarose gel.

### 2-The Quantitative Real Time-PCR

The extracted RNA from the treated and non treated cell lines was used as template to examine the expression level of four different specific genes (P53, Bcl2, TNF-α) in the presence of housekeeping gene primers (GPDH). The Real time reaction consists of 12.5 µl of 2X Quantitech SYBR® Green RT Mix (Fermentaz, USA), 2 µl of the extracted RNA (50ng/ µl), 1µl of

**Table 1.** Oligo-nucleotide primer sequences used in this study

Primer name	Primer sequence from 5'-3'	Annealing Temperature
P53	AGGGATACTATTCAGCCCCGAGGTG ACTGCCACTCCTTGCCCCATTC	64°C
Bcl2	ATGTGTGTGGAGAGCGTCAACC TGAGCAGAGTCTTCAGAGACAGCC	63°C
IL-4	CTATTAATGGGTCTCACCTCCCAACT CATAATCGTCTTTAGCCTTTCCAAG	60°C
IL-6	GCCTTCGGTCCAGTTGCCTT GCAGAATGAGATGAGTTGTC	56°C
TNF-	TCTCTAATCAGCCCTCTGGCC TGGGCTACAGGCTTGTCACTC	60°C
IL-2	CAGCCTTGCAGAAAAGAGAGC CCAGTAAGGCCAGGCAACAT	65°C
GPDH (House keeping gene)	ATTGACCACTACCTGGGCAA GAGATACACTTCAACACTTTGACCT	65°C

25 pM/ $\mu$ l forward primer, 1  $\mu$ l of 25 pM/ $\mu$ l reverse primer (Table 1), 9.5  $\mu$ l of RNase free water for a total of 25  $\mu$ l. Samples were spun before loading in the rotor's wells. The real time PCR program was performed as follows: initial denaturation at 95 °C for 10 min.; 40 cycles of 95°C for 15 sec, annealing at (Table 1) for 30 sec and extension at 72 °C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene 6000 system (Qiagen, USA).

### Data Analysis

Comparative quantitation analysis was done using Rotor-Gene-6000 Series Software based on the following equation:

$$\text{Ratio target gene expression} = \frac{\text{Fold change in target gene expression} \left( \frac{\text{expt}}{\text{control}} \right)}{\text{Fold change in reference gene expression} \left( \frac{\text{expt}}{\text{control}} \right)}$$

The data set of both samples and control of real-time PCR was analyzed with appropriate bioinformatics and statistical program for estimation of the relative expression of genes using Real Time PCR and the results normalized to GPDH gene (Reference gene). The data were statistically evaluated, interpreted and analyzed using Rotor-Gene-6000 version 1.7.

## RESULTS

### Methanolic extract yield and total phenolic content

The total phenolic compounds in the chichorium methanolic extract was 0.01075 in root but it was 0.00962 (mg/ml) in case of leaves. Additionally, when the

antioxidant activity of the root phenolic compounds was examined, the results revealed that the antioxidant activity increased by increase its concentration.

### HPLC analysis

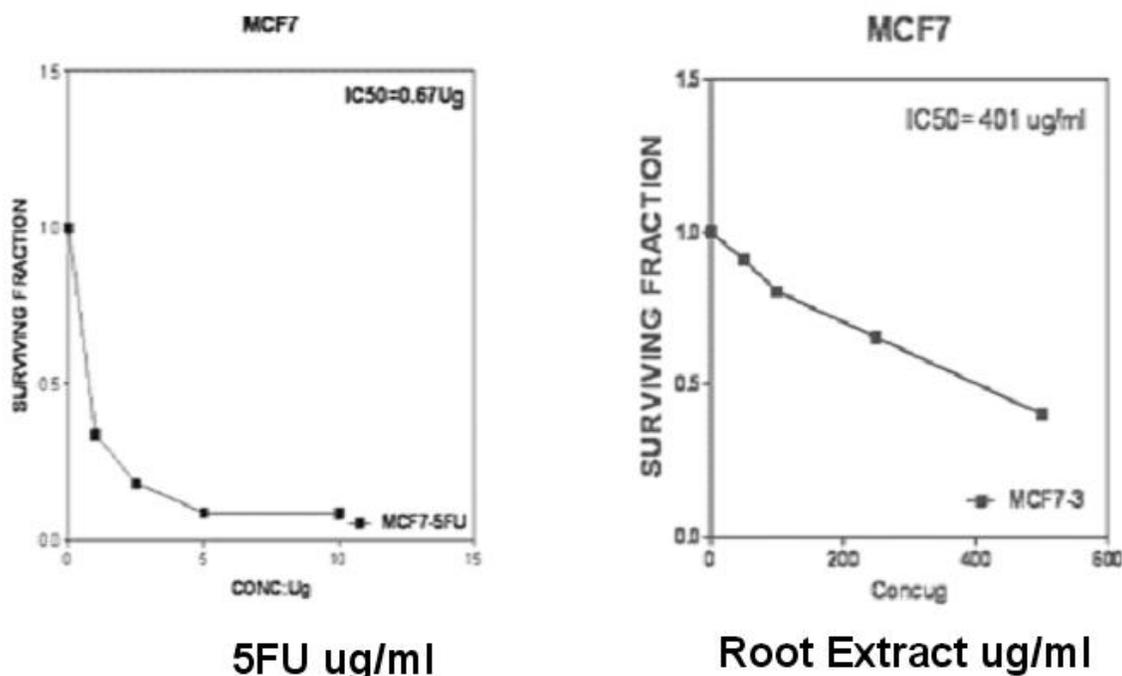
HPLC analysis of the phenolic compounds in the root extract revealed that the most abundant phenolic compounds were Catechin and pcoumaric acid as shown in Table 2. The results of chemical analyses were compared with those of standard solutions analyses, as explained in the experimental section.

### The root extracts and breast cancer cell line cytotoxicity Cytotoxicity of plant root extract

The cytotoxicity of root extract on MCF7 cell line was assessed by a cell viability assay, in the presence of different concentrations of the compound for 24 h. Under these experimental conditions, root extract exhibited a significant cytotoxic effect on MCF7 cell. The IC<sub>50</sub> values for MCF7 were 401  $\mu$ g/ml interpolated from linear regression curves, respectively (Figure 1). But the 5FU IC<sub>50</sub> value was 0.67  $\mu$ g/ml as shown in (Figure 1A). Both of the 5FU and root extract showed high range of killing toward the examined cell line. On the other hand the best concentration of the plant extract was obtained 250  $\mu$ g/ml which killed about 78% of the treated cells. In the treated breast cancer cells the highest rate of killing (92%) was

**Table. 2.** HPLC analysis of the phenolic compounds in *Cichorium pumilum* root extract

Phenolic compounds	Concentration mg/ml
Catechin	41.96388
Pcoumaric acid	0.276594
Ferulic acid	0.000332722
Cinnamic acid	0.000256481
Sinapic acid	0.00363446



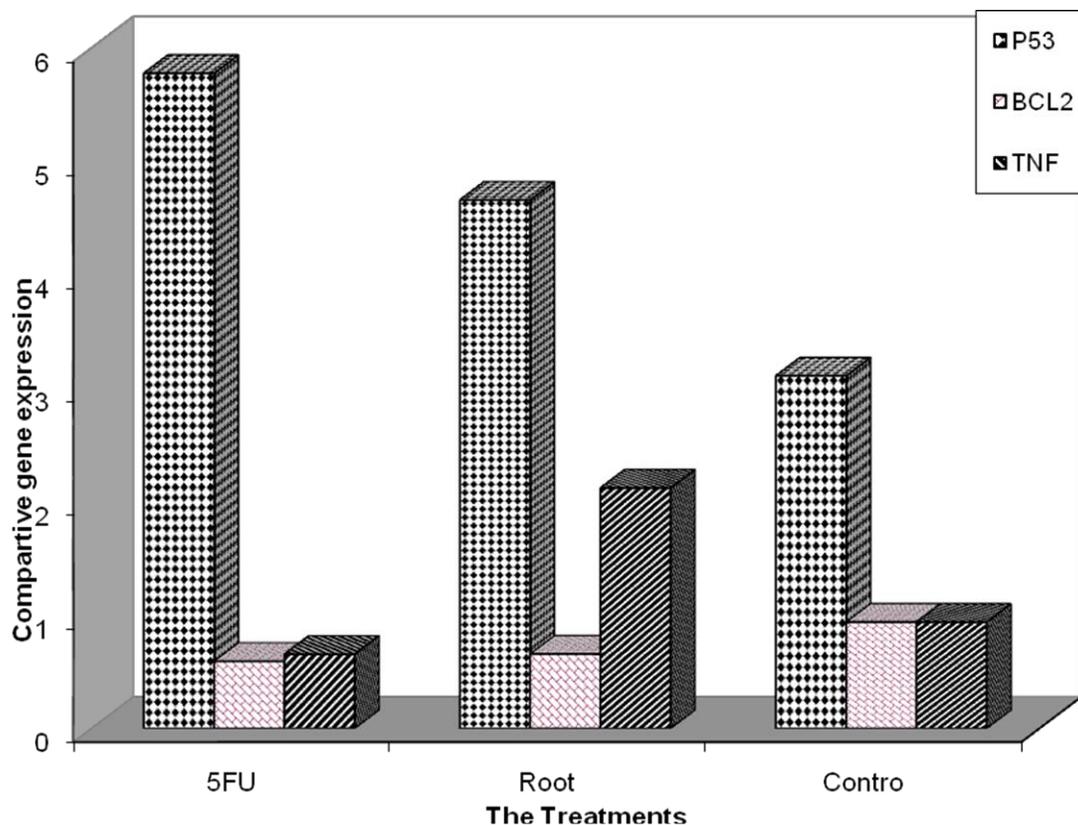
**Figure 1.** Cytotoxicity and IC50 of both 5 FU and Cichrium root extract on breast cancer cell lines (MCF7)

observed with concentration 5 and 10 ug/ml of 5 FU.

Results presented in Figure 3 revealed that expression level of p53 was so high with 5 FU but it decreased with the root extract and it was so low in the control ones. But in case of the BCl2 was so low but in cells treated with 5FU and root extract as well. In case of TNF the comparative expression was low in both cells treated with 5FU and the control ones but it was higher three times in the cells treated with the root extract. IL-2, IL-4 and IL-6 expression was so low in the cells treated with 5FU compared with the control cells. In contrast, cells treated with root extract the expression of IL2 was so high when compared with the control cells and the cells treated with 5 FU. But the expression of IL-4 was in equal to what obtained by the 5FU. IL-6 showed high expression three times more what obtained with 5FU and three times as in control cells.

**DISCUSSION**

Plants are used in the treatment of human diseases all the time. From the last century, a scientific interest for phytotherapy increased in several medical fields such as immunology, oncology, hematology and the use of plants in medicine has affected the identification of natural compounds: cocaine, morphine, vinblastine, taxolo, codeine are some examples Balunas and Kinghorn 2005). The *C. endivia* plant methanolic extracts were analyzed in HPLC and it was observed the most abundant pehnolic compounds were Catechin and pcoumaric acid with concentrations 41 and 0.27 mg/ml respectively. We used to use the cichurium as food in Arabian countries which pushed us to examine its activity as anticancer (Skaper, 1997). Steinmetz and Potter (1996) strongly suggest that vegetable-based diets



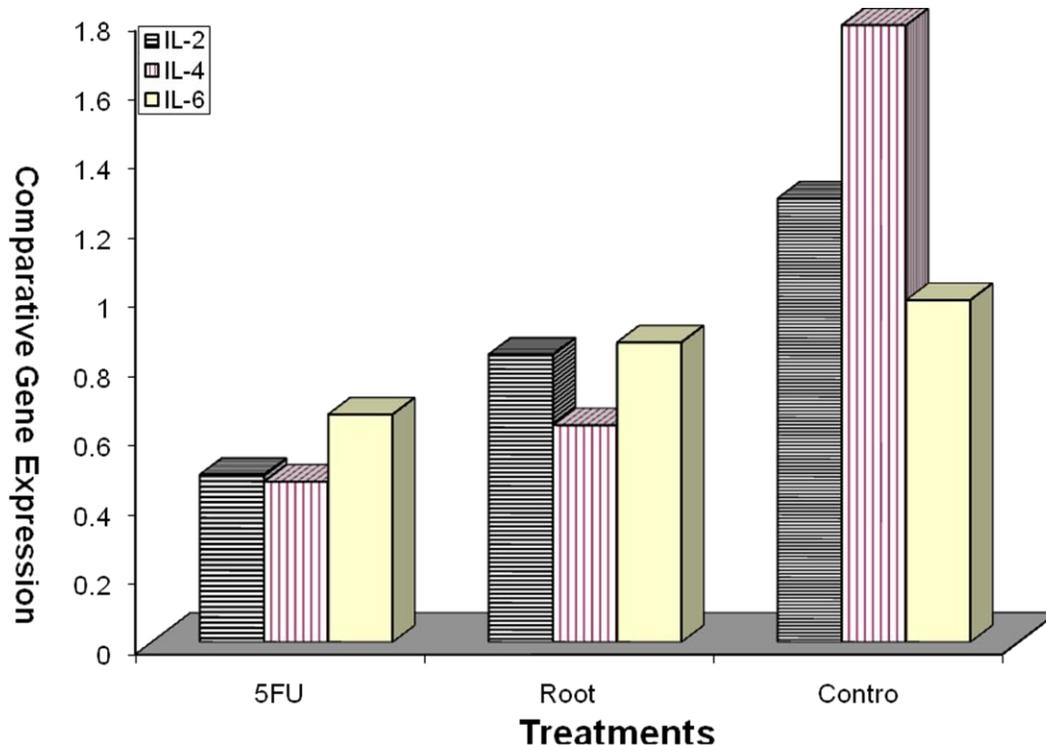
**Figure 2.** Comparative expression level for P53, Bcl2 and TNF genes in breast cancer cell lines (MCF7) treated with 5FU, cichrium root extract and control untreated breast cancer cells using Real Time PCR

contain, besides all the traditional nutrients, other substances that reduce the risk of cancer. Among the properties of phenolic compounds, they have been found to protect plants against oxidative damage and may have the same role in humans (Ruffa et al., 2002 and Rodriguez-Vicente et al., 1998). They have a wide range of action, which includes antitumoral, antiviral, antibacterial, cardioprotective, and antimutagenic activities (Rice-Evans et al., 1969 and Sergediene et al., 1990). They may act in different stages of the development of malignant tumors by protecting the DNA from oxidative damage. They inactivate carcinogens by inhibiting the expression of mutagenic genes; they also inactivate the enzymes charged with activating procarcinogens and activate the systems responsible for the detoxification of xenobiotics (Bravo, 1998). Our results come in agreement with that obtained by Rodriguez-Vicente *et al.*, (1998), who examined cell lines which showed different degrees of sensitivity to the compounds assayed. The lower resistance of these cells and the greater resistance of chichurium phenolic compounds seem to be related to the characteristics of breast cancer cell line and perhaps their origin because the former corresponds to a primary tumor culture and the latter to a metastatic cell. Phenolic compounds

activity was examined on thyroid carcinoma cell lines (Yin et al., 1999), breast (Wang and Kurzer, 1997), and cervix, and non-small-cell lung carcinoma (Caltagirone et al., 1997) and (Kudo et al., 1999); however, few studies have been carried out with melanoma cultures (Martinez et al., 2003) and most of them are exclusively related to metastatic processes (Caltagirone et al., 2000).

The Bcl2 showed down regulation (low expression) in the treated cells with both 5FU and cichorium root extract when compared with none treated cells (control). The obtained results agree with that obtained by Yadav et al., (1992), who reported that a large portion of these nutraceuticals show great potential for targeting cancer through various mechanisms such as the anti-apoptotic proteins (e.g., bcl-2, bcl-xL), promoters of cell proliferation (e.g., cyclooxygenase-2 [COX-2], cyclin D1, c-myc), invasive and metastatic genes (e.g., matrix metalloproteinases [MMPs], intracellular adhesion molecule-1 (ICAM-1), and angiogenic protein (vascular endothelial growth factor (VEGF) (Melis et al. 1991).

Specifically, Bcl-2 is down-regulated and Bax is up-regulated in the sequence of events from endometrial hyperplasia to endometrial carcinoma (Wehrli et al., 1998, Kokawa et al., 2001; Bozdogan et al., 2003). Some authors have suggested that a frame shift mutation of



**Figure 3.** Real Comparative expression level for IL2-IL4 and IL-6 in genes breast cancer cell lines (MCF7) treated with 5 FU, cichrium root extract and control untreated breast cancer cells using Real Time PCR.

Bax may be involved in the genesis of endometrial carcinoma (Wehrli et al., 1998, Kokawa et al., 2001; Estevez et al., 2002; Pena-Reyes et al., 2000). Over expression of anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-XL have been implicated in cancer chemoresistance, whereas high levels of pro-apoptotic proteins such as Bax promotes apoptosis and sensitize tumor cells to various anticancer therapeutics (Pena-Reyes et al., 2000; Burke and Walker, 2003; Reed, 1997). However, most of the work correlating chemoresistance and chemoresponsiveness has been performed in cancer derived cell lines. In a study using ovarian cancer cell lines, Yang *et al.*, (2002) have reported that cisplatin resistance was associated with the overexpression of anti-apoptotic protein Bcl-2 and down regulation of caspase-3 activity, but not associated with the expression of Bax and Bcl-XL (Pena-Reyes et al., 2000; Burke and Walker, 2003; Reed, 1997). Doxorubicin has been shown to upregulate the expression of Bax protein and downregulate the expression of antiapoptotic Bcl-2 proteins (Leung and Wang, 1999). Similar results are also observed with paclitaxel which causes a decrease in Bcl-2 expression and increase in Bax expression (Tudor et al., 2000). As Bcl-2 down-regulation is regarded as a major factor of chemosensitivity, an antisense Bcl-2 (Genasense) is presently undergoing phase I/II clinical trials also in combination with taxanes

(Chi et al., 2002, Tolcher et al., 2005).

Although published evidence suggests the involvement of multiple genes important for the onset of growth inhibition and apoptosis, such as p21, p53, GADD genes, BCL-XL, BCL-2, BAX, and APO2/ tumor necrosis factor-related apoptosis-inducing ligand (Sarkar et al. 2002, Sauane et al., 2003). In the obtained results the common future is the increasing the expression of the p53 gene in treated cells compared with the control ones. The expression was high in cells treated with the 5FU more than the treated with plant extract and lesser in the control ones.. Under normal growth conditions, p53 is a short-lived protein and is expressed at relatively low basal levels within the cell. In response to various cellular stresses, including exposure to DNA-damaging agents, UV and irradiation, hypoxia, and nucleotide depletion, p53 is rapidly induced and functions as a transcriptional activator. Specifically, it binds to consensus p53 binding sites expressed on a host of target genes, such as p21, GADD45, cyclin G, IGFBP3, bcl-2, and bax, as well as a newly identified gene, p53AIP1 (Glaspy, 2002; Milowsky and Nanus, 2001). In response to genotoxic stress as observed after treatment with anticancer agents, p53 can act to arrest cell cycle progression and, in so doing, help to preserve the integrity of the cellular genome or may directly activate the process of programmed cell death. Recent studies have documented that the expression of

p53 is controlled by a translational autoregulatory feedback loop in which p53 protein interacts directly with a target sequence on its cognate p53 mRNA. The in vivo biological relevance of the p53 protein–p53 mRNA interaction has been further supported by the isolation of p53 protein–p53 mRNA complexes from whole-cell extracts of rat embryonic fibroblasts (Dumoutier et al., 2001)

The root extract of cichorium induced the expression of the TNF gene in breast cancer cell line but the expression was so low both in control cells followed by the treated with 5FU. Recent studies proved that the NF- $\kappa$ B, factors such as TNF and interleukins (IL-1 $\beta$ , IL-6, and IL-8) also serve as connecting links between inflammation and cancer. TNF is released mainly from macrophages and regulates immune cells. Its dysregulation and overproduction lead to cancer and other diseases. TNF also plays a role in the activation of NF- $\kappa$ B by binding to a TNF receptor present on the cell surface that in turn triggers a pathway that leads to the activation of IKK (Brabers and Nottet, 2006; Szelényi, 2001). Signaling through TNF or cachectin and its two receptors TNF-R1 (has a DD) and TNF-R2 (does not have a DD) can lead to two different outcomes, apoptosis or survival. The survival pathway is under the control of the transcription factors NF [47] and AP-1, which become activated after TNF engages either TNF-R. Most of the effects of TNF are achieved through TNF-R1 engagement, although TNF-R2 does enhance TNF-R1 signaling (Van Antwerp et al. 1996). TNF-R1 directly interacts with TRADD via their DDs. TRADD is an adaptor where different signaling intermediates can bind and lead to death or survival pathway activation. TRADD (Hsu et al., 1995) facilitates the interaction between the TNF receptor and FADD, activating apoptosis. Once FADD is recruited to the receptor via TRADD, it uses its death effector domain (DED) to bind the DED of pro-caspase 8 (Chinnaiyan et al., 1995) which becomes activated and initiates the downstream caspase cleavage cascade.

The interleukins IL-2, IL-4 and IL-6 expression was lower in the cells treated with 5FU compared with the control cells but. On the other hand, IL-2 was expression in cells treated with cichorium root extract was higher more than 6 times more than the cells treated with 5FU and the control ones. In contrast, the expression of IL-4 in the cells treated with chicorium extract was in equall with the expression of IL-4 in the cells treated with 5FU and lower than the control cells. In case of IL-6, expression was high in cells treated with root extrac 4 times compared with that obtained with 5FU and 2 times more than that obtained with the control cells. These results agree with that obtained by Enk et al., (2004), who tested the ethanolic extracts of roots, stalks, and inflorescences of populations of wild *Cichorium endivia* subsp. *divaricatum* (Asteraceae) in terms of protection against sunburn, and in prevention of UVB-induced pyrimidine dimer formation

and IL-6 mRNA expression in the human keratinocyte cell line, HaCaT. Using ELISA technique for detection of pyrimidine dimers and RT-PCR for detection of IL-6, we found that the ethanolic extract of *C. endivia* roots absorbs radiation in the UVB spectrum and partially prevents induction of pyrimidine dimers and IL-6 expression. Interleukins are a group of cytokines released in the body from numerous cells in response to various stimuli. While IL-1 $\beta$  plays an important role in the inflammatory response against infection by increasing the expression of endothelial adhesion factors, thus allowing infiltration of leukocytes at the site of infection, IL-6 is a proinflammatory cytokine released in response to trauma or tissue damage. IL-8, a member of the CXC chemokine family also known as CXCL8, can function as a mitogenic, angiogenic, and mutagenic factor promoting cancer progression (Xie, 2001).

Platycodon also enhanced the mRNA expression of cytokines IL-2, IFN- $\gamma$ , IL-4, and IL-10 and transcription factors T-bet and GATA-3 in mice splenocyte induced by concanavalin A. It also suppressed TNF-induced apoptosis through the activation of NF- $\kappa$ B and NF- $\kappa$ B-regulated gene expression induced by carcinogens and inflammatory stimuli (Yadav, et al., 2010).

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