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Molecular and Biochemical Evaluation of Anti-Proliferative Effect of (*Cichorium endivia L.*) Phenolic Extractson Cancer Cell Line HCT-116

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Abstract: Cancer rates could further increase by 50% to 15 million new cases in the year 2020, according to the World Cancer Report, the most comprehensive global examination of the disease to date. Colorectal cancer (CRC) is a worldwide problem, with an annual incidence of approximately 1 million cases and an annual mortality of more than 500,000. Medicinal plants are considered to be the most hopeful way for cancer treatment. The *Cichorium endivia,*L.plant materials were collected from different regions in Tanuma, Saudi Arabia. Plant root water extract was performed and tested for toxicity. The anticancer activity of the plant root extract was examined on colon cancer cell lines HCT-116. The extract degrees of activity were measured by determining cytotoxcity for the cell line compared with anticancer drug 5 FU (5-fluorouracil)for long 24 hours.TheIC50was 0.78 ug/ml in case of the 5FU but 339 ug/ml with phenolic extract. The gene expression for the DNA cancer markers; P53 expression was the same in the control cells and the treated cells either by the plant root extract or the 5FU. Bcl2 showed low expression in the treated cells compared with the control but it was lower in cells treated with 5FU than the cells treated with root extract. TNF expression was the same in the cells treated with 5FU and the control one but the expression was so high in the cells treated with plant root extract. Interleukin IL-2, IL-4showed low expression in cells treated with plant root extract compared with the 5FU. But in case of IL-6cells treated with the plant root extract was so high compared with 5FU and the control cells. Thus, *Cichorium endivia*, which contains a combination of phenolic compounds, represents an enjoyable means of anticancer especially for colon cancer.

Key words: Colon Cancer · Real Time PCR · *Cichorium endivia* And Cancer Markers

INTRODUCTION

Natural products and related drugs are used to treat 87% of all categorized human diseases including bacterial infection, cancer and immunological disorders [1]. About 25% of prescribed drugs in the world originate from plants [2] and over 3000 species of plants have been reported to have anticancer properties [3]. About 80% of the population in developing countries relies on traditional plant based medicines for their primary health care needs [4]. Bangladesh has a rich and prestigious heritage of herbal medicines among the South Asian countries. More than 500 species of medicinal plants are estimated as growing in Bangladesh and about 250 species of them are used for the preparation of traditional medicines. However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compound(s) [5]. Traditional records and ecological diversity indicate that Bangladeshi plants represent an exciting resource for possible lead structures in drug design.

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 [6]. Enk *et al* .[7]had 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 dimmer formation and IL-6 mRNA expression in the human keratinocyte cell line, HaCaT.Enk *et al* .[7]they found that, by using ELISA technique, ethanolic extract of *C. endivia* roots absorbs radiation in the UVB spectrum and partially prevents induction of pyrimidine dimmers and IL-6 expression. They proved that application of the *Cichorium endivia* root extract on the skin prior to UVB irradiation totally prevented erythema.

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Hasan *et al*. [6] reported that to evaluate the plant's methanolic extract for *in vitro* cell growth supporting activity on rat hepatocytic 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 *Cichoriumintybus*Linn were screened for antihepatotoxic activity on carbon tetrachloride (CCl4)-induced liver damage in albino rats [8]. 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 antihepaticcompound (AB-IV) were found to possess a potent antihepatotoxic activity comparable to the standard drug Silymarin (Silybon-70) [8]. 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*. 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 Phenolic Contents from Plant Tissues: Total phenolic contents were assayed using the Folin–Ciocalteu reagent according to Dewanto *et al*. [9] aliquot of diluted plant root extractwas added to 0.5 ml of distilled water and 0.125 ml of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before addition of 1.25 ml of 7% Na_2CO_3. The solution was then adjusted with distilled water to a final volume of 3 ml and mixed thoroughly. After incubation in dark, the absorbance at 760 nm was read versus the prepared blank. Total phenolic contents of the root extractwere expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAEg-1 DW) through the calibration curve with gallic acid. All samples were analyzed in three replications.

HPLC Analysis: The root extract was subjected to the HPCL analysis according to Dittmann *et al*. [10]. 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 LiChrospher100 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 ul from the crude extract.

Human Tumor Cell Lines: Cell line:HCT-116 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/Lglutamine, 1.5 g/L sodium bicarbonate, 1.0 mM/L **nonessentialaminoacids**, 1.0 mmol/L sodium pyruvate andincubated at 37°Cin a humidified atmosphere containing 5% CO2.

Cytotoxicity Assay (MTT assay): The cytotoxicity assay was carried out as previouslydescribed by Balunas and Kinghorn, [11]. Briefly, between 3000 to 6000 cells, depending on the growth characteristics were seeded in each well of a 96 wellmicrotiterplate and incubated for 24 h at 37°C. Different concentrations of the plantroot extract 50, 100, 250 and 500 ug (in quadruplicates) wereadded to the exponentially growing cells. Cell controlsin absence of the compound were included with different concentrations of 5FU (1, 2.5, 5 and 10 ug). After anincubation period of 24 h at 37°C, the MTT assay was performed following the manufacturer's instructions. Theabsorbance values at 570 nm were recorded inan ELISA plate reader (Meterech 960). The percentage of living cells was calculated according to the following equations:

% of living cell= Absorbance of sample- Absorbance of DMSO (Blank)/ Absorbance of control- Absorbance of DMSO (Blank)100X. The IC50 for both cell lines were determinedby 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 Treaded Cell Lines

Extraction of Total RNA from Treated Cell Linefor 6 Hours: The best concentrations of the plant root extract which gave a high toxicity with the three examined colon

Table 1: The primers used in this study

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

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 10^6 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.

The Quantitative Real Time-PCR: The extracted RNA from the treated and non treatedcell lines was used as template to examine the expression level of four different specific genes (P53, Bcl2, TNF-γ) 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 25 pM/µl forward primer, 1 µl of 25 pM/µl reverse primer (Table 1), 9.5 µl of RNase free water for a total of 25 µl. 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 6000system (Qiagen, USA).

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

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

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 GPDHgene (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: Thetotal 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 root extract revealed that the most abundant pehnolic 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.

Table 2: HPLC analysis for the phenolic compounds in the plant extract

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

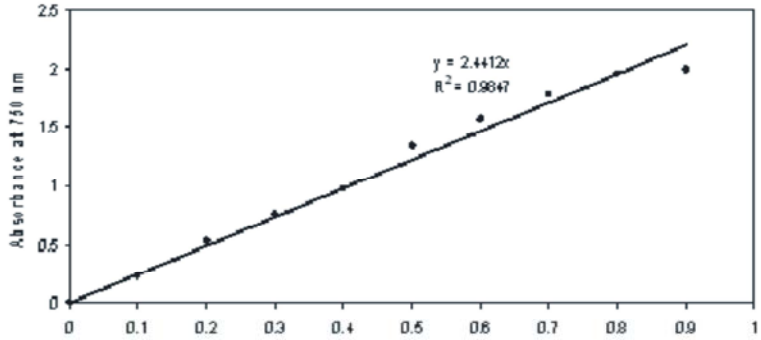

Fig. 1: Standard curve for Gallic acid and measuring the consequence phenolic concentrations.

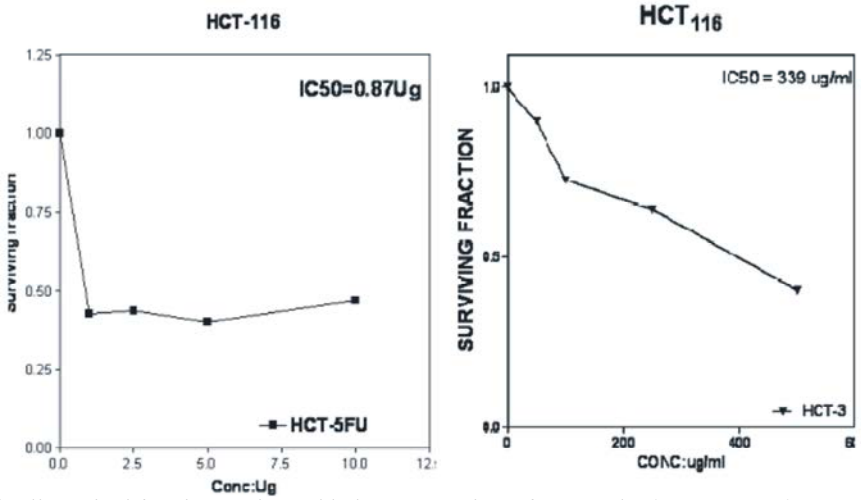

Fig. 2: A; B:.Plot of cell survival fraction against added concentration of 5Fu and *Chicorium endivia L* extract as applied on the Colon cancer (HCT116).

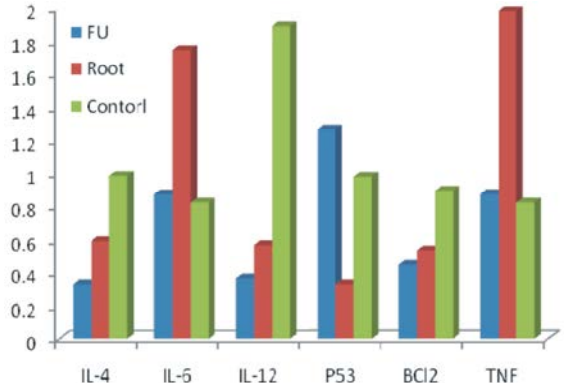

Fig. 3: Real Time PCR for different cytokines(IL2-IL6-IL4) and DNA cancer marker (P53,Bcl2, TNF) expression in cell line HCT116 treated with 5FU and Cichriumroot extract in 3 different

The Root Extracts and Cell Lines Cytotoxcity

Cytotoxitcity of Phenolic Root Extract: The cytotoxicity of root cichurium root extract on HCT-116 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 HCT-116 cells. The IC50 values for HCT-116 was 339ug/ml interpolated from linear regression curves (Fig. 1). Also the results were compared with the 5FU and the IC50 values for HCT-116 was 0.87 as shown in figure (2A). Both of the 5FU and root extract showed high range of killing toward the examined cell line.The highestkill rate for the HCT-116 was observed in the cells treated with 5 ug/ml of 5FU which killed 60% of the treated cells. The same percentage killed cells was obtained by the root extract concentration 500ug/ml.

The real time PCR analysis revealed that, P53 expression was the same in the control cells and the treated cells either by the plant root extract or the 5FU. The Bcl2 gene showed low expression in the treated cells compared with the control but it was lower in cells treated with 5FU than the cells treated with root extract. TNF expression was the same in the cells treated with 5FU and the control one but the expression was so high

in the cells treated with plant root extract. Interleukin IL-2, IL-4 showed low expression in cells treated with plant root extract compared with the 5FU. But in case of IL-6 cells treated with the plant root extract was so high compared with 5FU and the control cells. Thus, *Cichorium endivia*, which contains a combination of phenolic compounds, represents an enjoyable means of anticancer especially for colon cancer.

DISCUSSION

Cichorium endivia family Asteraceae, is growing naturally Aseer region. 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 [11]. When the cicurium was subjected to methanolic extraction it was observed the most abundant pehnoliccompounds wereCatechin and pcoumaric acid with concentrations 41 and 0.27 mg/ml respectively.

It was known from previous studies that phenolic compounds have anticancer activity [12]. Steinmetz and Potter [13] strongly suggest that vegetable-based diets 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 [14, 15], they have a wide range of action, which includes antitumoral, antiviral, antibacterial, cardioprotective and antimutagenic activities [16-18]. Phenolic compounds activity was examined on thyroid carcinoma cell lines [20], breast [21] and cervix and non-small-cell lung carcinoma [22, 23], however, few studies have been carried out with melanoma cultures [24] and most of them are exclusively related to metastatic processes [25].

For examining the effect of the *Cichorium* root extract on the colon cancer cell line, the gene expression of the Bcl2 was tested using real time PCR, it was observed a down regulation in Bcl2 expression in the examined cell lines. On the other hand a high Bcl2 gene expression was shown in the cells when treated with the 5FU. We assumed that root extract and especially its penolic compounds helped insupressing of the Bcl2 in the treated cells with root extract. Theseresults agree with that obtained by [26-30], they reported that a large portion of these nutraceuticals show great potential for targeting cancer through various mechanisms such as the downregulation of transcription factors (e.g., nuclear factor-kappaB [NF-κB]), 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) [31-36]. Similar results are also observedwith paclitaxel which causes a decrease in Bcl-2 expressionand increase in Bax expression [37-39].

TheP^{53} is a critical cellular protein that plays an essential role in regulating the cell cycle and the cellular response to certain environmental and/or genotoxic stresses [40, 41]. 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 transciptional 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 [42, 43]. Recent studies have documented that the expression of p53 iscontrolled 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 [44].

We suggest that the phenolic compounds involved in the plant root extract may induce the expression of the TNF gene in the treated cell lines. On the other hand the expression of the TNF in the cells treated with 5FU was in the same level for the control cells. Recent studies proved that the NF-κB, factors such as TNF and interleukins (IL-1β, IL-6 and IL-8) also serve asconnecting 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-κB by binding to a TNF receptor present on the cell surface that in turn triggers a pathway that leads to the activation of IKK [45, 46]. Signaling through TNF or cachectin and its two receptors TNF-R1 and TNF-R2 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 [48, 49].

Both Interlukins IL-2 and IL-4 expression decreased in the treated cell line with the plant root extract and with 5FU. The expression was lower in the cells treated with 5FU than that treated with the phenolic compounds. In case of IL-6, expression was highin cells treated with phenolic compounds but it was low both in the control and 5FU treated cells. These results agree with that obtained by Enk *et al.* [7], 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-1β 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 [50]Platycodon also enhanced the mRNA expression of cytokines IL-2, IFN-ã, 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-κB and NF-κB-regulated gene expression induced by carcinogens and inflammatory stimuli [51].

CONCLUSION

It can be concluded that the wild plant *Cichorium endivia*, which contains a combination of phenolic compounds, represents an enjoyable means of anticancer especially for colon cancer.

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