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Research Paper

EXPRESSION OF TUMOR-MARKERS AND CYTOKINES IN RESPONSE TO *CICHORIUM ENDIVIA* L. IN CANCEROUS MICE

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Most of natural products, such as, medicinal plants and herbs confer protective effects against a wide range of cancers, including colon cancer. *Cichorium endivia*, L. has been shown to have anti-inflammatory and antioxidant properties. Thirty two male mice were divided into four groups. Group I served as control. Groups 2, 3 and 4 were administered freshly prepared DMH for three weeks, twice per week. Group 3 received the anticancer drug 5 FU for two weeks. Half of this group was sacrificed after 2 weeks while the other half continued until the experiment ended. Group 4 received the plant extract which was divided into 3 sub-groups. Each sub group was administered with a different concentration of the extract (200-400-600 mg/kg bodyweight/ day) for 40 days. Animals were sacrificed; blood was collected and the colons were subjected for investigation. P53 expression was nearly the same in both control and all treated tissues. Bcl₂ expression decreased in mice treated with 5FU and specifically with Cichorium. TNF- α expression was lower in tissues treated with 5FU compared with the control. Meanwhile, the expression of dose 200 mg/kg.b.wt, was lower than both the control and the 5FU treated tissues. The real time PCR analysis of blood samples showed that the expression of interleukin IL-12 and IL-4 was higher in blood cells treated with plant extract compared with the 5 FU. The two effective doses were 200 and 600 mg/kg.b.wt. In contrast, therapeutic treatments mute the expression of interferon IFN marker. It is thus concluded that, *Cichorium endivia*, especially dose 200 mg/kg.b.wt., represents an effective anticancer and immune response drug especially for colon cancer.

Keywords: *Cichorium endivia*, Tumor-markers, P53, Bcl₂, TNF- α , Cytokines, IL-12, IL-4, IFN

INTRODUCTION

Colon cancer (CRC) is one of the most common cancers in the developed countries. Due to the

limited prevention and treatment options, colon cancer is considered to be one of the major causes of cancer-related death (Alshehri, 2012).

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1, 2- Dimethyl hydrazine (DMH) has been shown to induce colonic carcinomas in rats and mice (Sitohy and El-Salhy, 2001). Natural products and related drugs are used to treat 87% of all categorized human diseases including cancer and immunological disorders (Newman and Cragg, 2007). Over 3000 species of plants have been reported to have anticancer properties. About 80% of the population in the developing countries relies on traditional plant-based medicines for their primary health-care needs. *Cichorium sp.* belongs to the family Asteraceae and it is a small aromatic biennial herb. The whole plant contains a number of medicinally important compounds such as inulin, esculin, volatile compounds, coumarins, flavonoids and vitamins (Alshehri and Hafez, 2012).

Cichorium endivia had been tested in terms of prevention of ultraviolet B (UVB)-induced pyrimidine dimer formation and interleukin-6 (IL-6) mRNA expression in the human keratinocyte cell line, HaCaT. They found that ethanolic extract of *C. endivia* roots absorbed radiation in the UVB spectrum and partially prevented induction IL-6 expression. They proved that application of the *Cichorium endivia* root extract on the skin prior to UVB irradiation totally prevented erythema (Enk et al., 2004).

The effect of hydro-alcoholic extract of *Cichorium endivia* L. leaves (HCE) has been elucidated against acetaminophen-induced oxidative stress and hepatotoxicity in male rats. The hepatoprotective activity with *C. endivia* leaves-extract in rats was found to be compatible with the known hepatoprotective drug "Silymarin" (Marzouk et al., 2011).

The gene expression of breast cancer cell line (MCF7) have been examined for the DNA cancer

markers; P⁵³, BCl₂, TNF- α and interleukin markers IL-4, IL-6 and IL-2, treated with the root extracts of *Cichorium endivia*. They found that due to all expressions of those markers, *C. endivia* showed to be anticancerous (Alshehri and Hafez, 2012).

The aim of the present work was to investigate *Cichorium endivia* as an anticancer agent and an immune response drug especially on CRC.

MATERIALS AND METHODS

Plant Materials

The *Cichorium endivia*, L. plant material were collected from the faculty of Agriculture farm, Alexandria University. The whole plants were washed with distilled water, and ground using a blender.

Chemical Materials

DMH (Sigma) was dissolved in double distilled water and adjusted to pH 6.5 (Sumiyoshi and Wargovich, 1990). 5-FU, Sigma was diluted in pyrogen-free 0.85% saline, to be at a concentration of 100 mg/10 mL (Kojima et al., 1999).

Experimental Design

Thirty two male albino mice with initial weight of (25-30 g) were used in all experiments. Mice were housed in plastic cages with filter tops (five per cage) under controlled conditions of light, humidity and temperature at the Animal House Lab., Medical Research Institute, Alexandria University. They were divided randomly into four groups. The 1st group (n=4) served as control. The 2nd group (n=8) was separated into two subgroups. Mice of the second group were injected intraperitoneal (i.p) of DMH at a dosage of 20 mg/kg b.wt. twice per week. Subgroup no.1 was treated for only three weeks (DMH stopped), while subgroup no.2

was treated continuously till the end of the experiment (DMH continued). The 3rd group (n=8) was treated with DMH for three weeks, then received 5-fu (80 mg/kg b.wt.) for two weeks. This third group was then separated into two subgroups. The first subgroup was scarified (5-FU stopped) while the second subgroup were kept without any treatment till the end of the experiment (5-FU continued). Mice of forth group were treated by DMH for three weeks, twice per week. The individuals of this group were divided to three subgroups. Each subgroup received a different concentration of *Cichorium* extracts 200, 400, 600 mg/kg bodyweight/ day for 40 days. All animals, except those of 5-FU stopped sub-group, were sacrificed after 9 weeks from the beginning of the experiment.

Real time PCR for Cancer DNA Markers and Immune Response Markers

Cancer DNA markers (p⁵³, Bcl₂ and TNF-a) were carried out on colon tissue which was removed and washed twice with ice-cold saline solution. The immune response markers (IL-4, IL-12 and IFN) was carried on from blood samples which was collected in EDTA coated tubes, centrifuged at 2000xg for 10 min to separate plasma and stored at -80°C until analysis.

Extraction of Total RNA

The RNA extraction was performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Extracted RNA was dissolved in DEPC-treated water and analyzed on 2% agarose gel. Stored at -80°C until used.

The Quantitative Real Time-PCR

The extracted RNA from colon tissues was used as template to examine the expression level of

three different specific genes (P⁵³, Bcl₂, TNF-α) in the presence of housekeeping gene primers (GPDH). While the RNA extracts from the blood samples was used to examine the expression level of three different specific genes (IL-4, IL-12, and IFN) also in the presence of housekeeping gene primers (GPDH). All RNA samples were treated with DNaseI to remove residual DNA. The reverse transcription from mRNA to complementary DNA (cDNA) was made using 4 μL of RNA per sample, 2.5 μL Buffer enzyme, 5.5 μL oligo d (t), 2.5 μL dNTPs, 0.2 μL taq enzyme and 5.3 μL RNase-free water. The mixture was incubated in a thermocycler at 25°C for 5 min. followed by 42°C for 60 min. and 70°C for 15 min. The Real time reaction consists of 12.5 μL for 2X Green Dye RT Mix (ROVALAB), 2 μL of the extracted cDNA, 1 μL of 25 pM/μl forward primer, 1 μl of 25 pM/μl reverse primer (Table 1), 9.5 μl of

Primer Name	Primer Sequence from 5' to 3'
TNF-α	F TTC TGT CTA CTG AAC TTC GGG GTG ATG GGT CC
	R GTA TGA GAT AGC AAA TCG GCT GAC GGT GGT GG
BCL ₂	F ATG TGT GTG GAG AGC GTC AAC C
	R TGA GCA GAG TCT TCA GAG ACA GCC
p ⁵³	F AGG GAT ACT ATT CAG CCC GAG GTG
	R ACT GCC ACT CCT TGC CCC ATT C
IL-4	F TCT GTG GTG TTC TTC GTT GC
	R TCA ACC CCC AGC TAC TTG TC
IL-12	F AAC TGG AGG GAG GAG TAC GGA GAA TGG
	R GGA AGC ACG GCA GCA GAA TA
IFN	F GTC ACA GTT TTC AGG TGT ATA GGG
	R AGC GGC TGA CTG AAC TCA GAT TGA AG
GAPDH	F ATT GAC CAC TAC CTG GGC AA
	R GAG ATA CAC TTC AAC ACT TTG ACC
OLIGO d(t)	(t)12-18

RNase-free water to make a total of 25 μ l. Samples were spun before loading in the rotors wells. The real time PCR program was performed as follows: initial denaturation at 95°C for 15 min; 45 cycles of 96°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s (Alshehri and Hafez, 2012). Data acquisition was performed during the extension step. This reaction was performed using Rotor-Gene 6000 system (Qiagen, USA).

DATA ANALYSIS

The data set of both samples and control for real-time PCR was analyzed with Rotor-Gene-6000 version 1.7.94. for estimation of the relative expression of genes using Real Time PCR and the results normalized to GPDH gene (Reference gene). Comparative quantitation were statistically evaluated, interpreted and analyzed by Livak and Schmittgen (2001).

RESULTS

The real time PCR analysis of colon tissue revealed that P⁵³ expression was nearly the same in the control and the treated tissues, except in the stopped 5-fluorouracil (5FU) where it was low (Figure 1). The Bcl₂ gene showed low expression in the treated tissues compared with the control, especially in tissues treated with plant extract (Figure 2). TNF- α expression was lower in tissues treated with 5 FU than the control. Meanwhile the TNF- α expression was so high in the tissues treated with plant extract at the two doses 400 and 600 mg/kg.b.wt, but the expression at the dose 200 mg/kg.b.wt was very low (Figure 3).

The real time PCR analysis of blood samples revealed that interleukin IL-12 and IL-4 showed

Figure 1: Real Time PCR for the DNA cancer marker, P⁵³, expression in colon tissue treated with 5FU and different doses of *Cichorium* extract

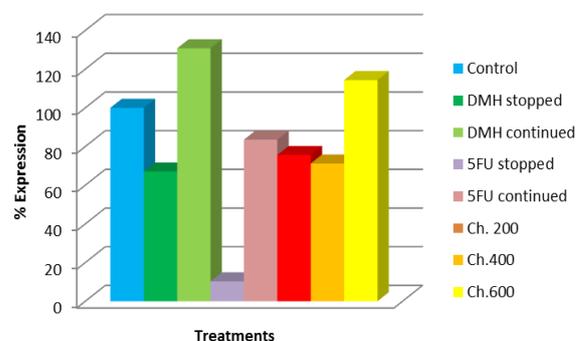


Figure 2: Real Time PCR for the DNA cancer marker, Bcl₂, expression in colon tissue treated with 5FU and different doses of *Cichorium* extract

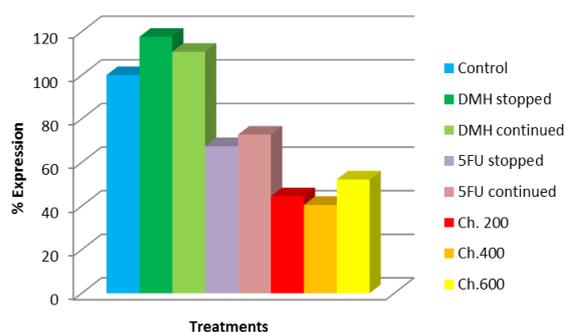
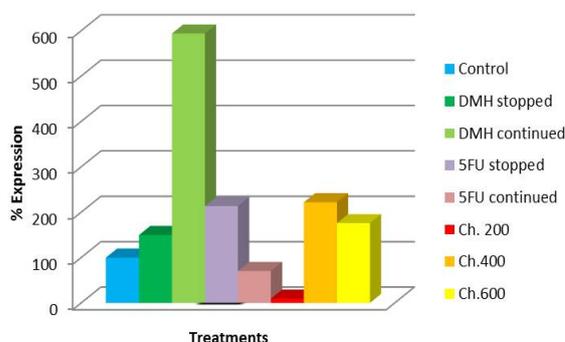


Figure 3: Real Time PCR for the DNA cancer marker, TNF- α , expression in colon tissue treated with 5FU and different doses of *Cichorium* extract



high expression in samples treated with 200 and 600 mg/kg.b.wt of plant extract compared with 5 FU (Figures 4 and 5). Whereas, 400 mg/kg.b.wt of plant extract didn't show expression of interleukins. IFN marker exhibited extremely low expression for 200 and 400 mg/kg.b.wt of plant extract and 5FU treatments compared with the control. But the expression of the dose 600 mg/kg.b.wt was high (Figure 6).

The results thus show that, *Cichorium endivia*, especially dose 200 mg/kg.b.wt, represents an effective means for anticancer activity as well as an immune response drug especially for *Colonic carcinoma*.

Figure 4: Real Time PCR for cytokines, IL-12, expression in blood samples from mice treated with 5FU and different doses *Cichorium* extract

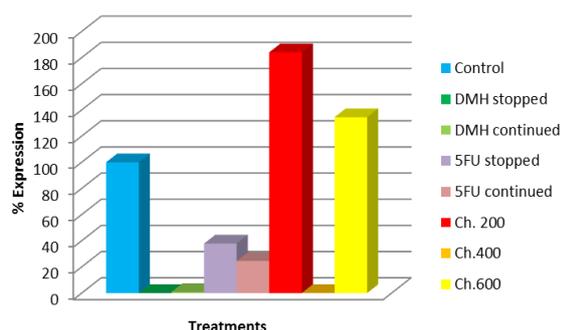


Figure 5: Real Time PCR for cytokines, IL-4, expression in blood samples from mice treated with 5FU and different doses *Cichorium* extract

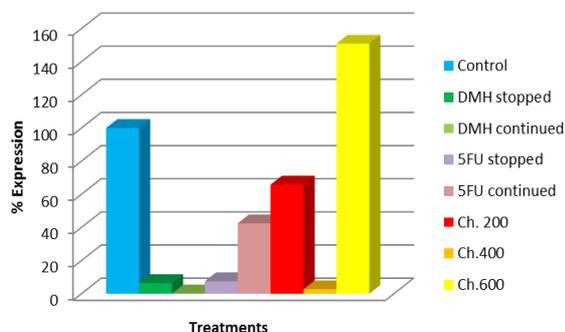
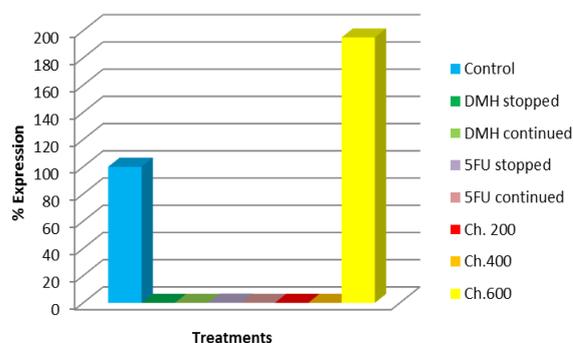


Figure 6: Real Time PCR for cytokines, IFN, expression in blood samples from mice treated with 5FU and different doses *Cichorium* extract



DISCUSSION

Colorectal cancer is the third most commonly diagnosed cancer in the world, but it is more common in the developed countries (Ferlay et al., 2010). Organic compounds from plants have extensive past and present use in the treatment of many diseases. Natural compounds from plants having antioxidant and immuno-modulatory activities have potential as therapeutic agents (Devasagayam and Sainis, 2002).

Family Asteraceae contained medically important plants such as *Cichorium endivia*, due to their polyphonic components. The expression of tumor suppressor gene (P^{53}) showed no variability in treated and control tissues. P^{53} can act to arrest cell cycle progression, help to preserve the integrity of the cellular genome and activate directly the process of programmed cell death. Some studies obtained similar results (Alshehri and Hafez, 2012).

The effect of *Cichorium* extract on colon cancerous tissues decreased the expression of the apoptosis regulator protein (BCI_2), even more than 5FU, compared with the control tissues. We assume that plant extract especially its phenolic

compounds play a role in decreasing BCl₂ in treated tissues. These results agree with those obtained by others [Sitohy and El-Salhy (2001); Kokawa *et al.* (2001) and Tudor *et al.* (2000)]. They reported that a large portion of these plants show great potential for targeting cancer through the down regulation of anti-apoptotic proteins (e.g., bcl-2, bcl-xL).

On the other hand, the expression of tumor necrosis factor alpha TNF- α in tissues treated with 5 FU and 200 mg/kg.b.wt was lower than the control tissues, with the plant extract treatment being lower than 5 FU treatment. Recent studies proved that the TNF-a levels increase in cancer cells with end-stage disease (Nakashima *et al.*, 1998).

Both Interleukins IL-12 and IL-4 expression increased in the treated blood cells with plant extract than with 5 FU. In case of interferon IFN marker, the plant extract and the 5 FU treated were extremely low compared with the control. These results agree with several previous studies [(Milowsky and Nanus (2001); Glaspy (2002) and Yang *et al.* (2011)]. They reported that interleukins 12 and 4 induces interferon γ secretion by T cells and natural killer cells. The above process enhances the proliferation of activated T cells and natural killer cells. IFN upgraded the apoptosis rate. Interleukin 12, 4 stimulates *In vivo* antitumor activity in many murine tumor models.

CONCLUSION

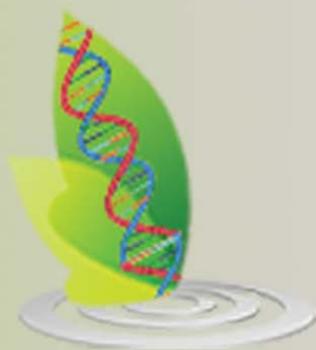
The present findings imply that the promising data obtained with *Cichorium endivia* in the pre-clinical models of anti-tumor immunotherapy have raised much hope that this could be a powerful therapeutic agent against cancer and immune reactions. However, the effective dose of 200 mg/

kg.b.wt needs further studies to reach the exact optimum dose.

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