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RESEARCH ARTICLE

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The Effect of Hydroalcoholic Extract of Amaranthus Retroflexus L. Seeds on Cox2 and Her2 Genes on the Induced Colorectal Cancer in an Animal Model of Balbc Mouse

Zahra Dorostgo, Hossein Movafagh, Reza Sarravi, Samira Abedi Araei, Fatereh Rezaei*
Department of Biology, Islamic Azad University, Babol Branch, Babol, Iran

ABSTRACT

Background: for many centuries, herbs and herbal extracts have been used as a treatment for diseases. Today, many medicines are derived from herbal sources. Herbs for treatment purposes are the source of many modern medicine treatments.

Materials and Methods: In this experimental study, the hydroalcoholic extract of *Amaranthus Retroflexus* L. seeds was prepared, then the required concentrations for the experimental stages were prepared and MTT test was performed on the CT26 and MRC5 cells. Simultaneously, tumor induction was performed in mice. The clone tissue was sampled, RNA was extracted, and finally, the cDNA was implemented. The results of gene expression changes were evaluated by RealTime technique with the t-test and ANOVA.

Results: *Amaranthus Retroflexus* L. seed extract reduced the survival and proliferation of cancer cells. The decrease in cell survival depends on concentration, and this decrease is significant ($p < 0.05$). Moreover, Cox2 and Her2 gene expression increased significantly on day 28 and then significantly decreased after the treatment with the extract ($p < 0.05$).

Conclusion: Treatment with *Amaranthus Retroflexus* L. seed extract decreased gene expression and cell proliferation in cell lines. Therefore, the extract of this plant can be used to improve cell proliferation and cancer cells.

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Amaranthus Retroflexus L., Cox2, Her2, MTT, Clone Cancer.

INTRODUCTION

Colorectal cancer is the third most common type of cancer in oncological pathology. It is currently the most common malignant cancer of the gastrointestinal tract, accounting for 13% of all malignant tumors, and is the second leading cause of cancer death in men and women in developed and underdeveloped countries. It is predicted that it would overtake the heart disease mortality in the coming years (Granados-Romero et al., 2017). Resistance to chemotherapy drugs, clarification of the mechanisms responsible for the growth and development of colon cancer, and the development of early and effective diagnostic strategies and reasonable treatment strategies have always been the focus of research on colorectal cancer (Sideris et al., 2014).

The diversity and prevalence of cancer over the years has led to a variety of treatments; however, despite many efforts to prevent and treat cancer, the disease develops and remains a leading cause of death in the world (Fleet et al., 2012).

The use of herbs for therapeutic purposes dates back to prehistoric times and is the source of many modern medicine treatments. Many common medicines are derived from herbal sources (Aiello et al., 2019).

Amaranthus Retroflexus L. is from the Amaranthaceae family that has four carbons, which is herbal, single-

* Contact Fatereh Rezaei, Department of Biology, Islamic Azad University, Babol Branch, Babol, Iran
faterehrezaei62@gmail.com

stemmed, and with an upright stem in red. The compounds in *Amaranthus Retroflexus* L. have given high antioxidant properties to this plant (Dino et al., 2017).

There is a need for basic research to study the molecular pathways in the pathogenesis of diseases and cancers (Shelton et al., 2010).

Cell proliferation is central to tumorigenesis, and cyclooxygenases (COXs) are important regulatory enzymes in this process. These regulatory compounds play an important role in various biological processes including cell proliferation, angiogenesis, immune function, and inflammation, all of which are important in the growth and development of neoplasms (Negi et al., 2019).

Cyclooxygenase (Cox) is a key enzyme in the conversion of arachidonic acid to prostaglandins. In mammals, there are two isoforms of this enzyme, called Cox1 and Cox2, with independent genes and different expression patterns. Cox1 enzyme is expressed continuously in most tissues, while Cox2 is rapidly induced as part of inflammatory reactions in response to extracellular stimuli, and its extent is immeasurable in most normal tissues. In addition, Cox2 plays an important role in regulating cell proliferation, eliminating differentiation, and causing carcinogenicity. Cox2 is associated with inflammation, pain, angiogenesis, and cancer (Negi et al., 2019). COX-2 is of particular importance because COX-2specific inhibitors such as nonsteroidal anti-inflammatory drugs (NSAIDs) including nonsteroidal anti-inflammatory drugs (NSAIDs) have been developed or are being developed that may play a role in chemical prevention of gastrointestinal neoplasms (Akbari et al., 2020).

Studies have shown that COX-2 expression is not regulated in precancerous lesions and precancerous carcinoma; it is positively correlated with tumor invasion and lymphatic metastasis. Therefore, increased COX-2 expression may occur in the early stages of the tumor, and COX-2 level detection is useful for early detection of CRC (Sheng et al., 2020).

The human epidermal growth factor receptor (Her2) represents a promising therapeutic target. This gene is located on the long arm of human chromosome 17 (17q12) and involved in cell proliferation and differentiation. The Her2 protein is a membrane receptor tyrosine kinase and a member of the epidermal growth factor receptor family (Ahn et al., 2020). This receptor is expressed in most tissues and plays a key role in various functions such as cell growth, proliferation, and differentiation. The purpose of this study is to use natural and herbal treatment to combat clone cancer.

Figure 1: RNA sample extracted on agarose gel

MATERIALS AND METHODS

This experimental study was performed in 2019 to investigate the effect of hydroalcoholic extract of *Amaranthus retroflexus* L. on Cox2 and Her2 genes on colorectal cancer induced in the animal model of Balbc mice. Accordingly, the *Amaranthus retroflexus* L. seeds were collected from Klagarkola village of Bandapi section of eastern Babol located in the north of Iran between July and August 2017, and the herbarium was determined by a botanist. The seeds of the plant were dried for 10 days at an artificial temperature of 40°C and crushed by an ordinary mill. Forty grams of the dried and powdered sample was extracted by the Soxhlet method with water and methanol solvent in a ratio of 20 to 80. The extract was concentrated by rotary evaporator and fully dried by a freeze dryer (Dryz dryer) completely as a powder. The yield of the extract was calculated and then different concentrations of the extract were used for treatment at different stages of the experiment. The CT-26 cancer cell line was purchased from the Cell Bank of the Pasteur Institute of Tehran and kept in the culture medium of Dulbeccos modified Eagles medium (DMEM) flasks containing 10% FBS and 1% penicillin-streptomycin antibiotics in 37°C incubator with 2% CO2. To perform tests, when the cells reached at least 70% cell growth, they were separated from the flask by Ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1500 rpm for 2 minutes. Cell precipitation was prepared in suspension in one cc of culture medium and the percentage of viability of cells in cell suspension was determined by mixing equal proportions of trypan blue using a hemocytometer slide and examination by light microscopy. After ensuring that the cells were not infected, they were used for testing with viability above 90%.

To evaluate the effect of *Amaranthus Retroflexus* L. seed extract on the growth and proliferation of cancer cells, the spectrophotometer method (3- (4,5-Dimethylthiazol-2-yl) -2,) (5-diphenyltetrazolium bromide, a yellow tetrazole MTT) was used. This method is a competitive mitochondrial metabolic test

and is based on the breakdown of tetrazolium salt by mitochondrial succinate dehydrogenase enzyme, in which 200 µl culture medium containing 10^5 cells were cultured in the 96-well plate. After 2h of incubation, the concentrations (31.25, 62.5, 125, 250, 500, and 1000) µg/ml of the extract were added to the cells and incubated for 4 hours. After this period, 20 µl MTT (Sigma Co. with 5 mg/ml c concentration) was added to each well and incubated in the dark for another four hours. Then the culture medium containing MMT was emptied carefully and 200 µl DMSO was added to each well to dissolve the purple formazan. After 15 minutes of incubation at room temperature, the light absorption of each well was read using an ELISA device at a wavelength of 100 nm. The results are reported based on the percentage of cell survival vs. the extract concentration.

$$Cell\ survival\ percentage = \frac{Optical\ absorption\ of\ test}{Optical\ absorption\ of\ control} \times 100$$

$$Cell\ survival\ percentage = \frac{absorption\ of\ specimen}{absorption\ of\ control} \times 100$$

Statistical analysis was performed using the SPSS software.

Simultaneously, tumor induction was performed in the left lateral region of balbc mice. To prevent the rejection of injected cancer cells, it was necessary to weaken and inhibit the immune system of mice. Therefore, 50 mg/kg cyclosporine body weight of the mice was used orally on a daily basis.

The experimental groups were:

In the next stage of the study, 24 mice were randomly divided into three groups (n = 8).

These three groups included the control group receiving PBS, the cancer group, and the cancer group receiving the *A.Retroflexus* L seed extract as treatment.

Clones were sampled from all the study groups. RNA was extracted from the prepared tissues using an extraction kit (Dena Zist Company, Iran). To evaluate the quality and quantity of RNA, agarose gel 1.5%, and a nanodrop device was used, respectively. Then, cDNA synthesis was performed for all samples using the Pars Toos kit.

For real time-PCR reaction, first cox2, Her2, and GAPDH gene primers were designed using oligo software version 7 and gene information in Gene Bank. The primers were then blasted in NCBI for confidence.

The primers used are listed in Table (1). The primers were then ordered from Gene Technologies. Real time-PCR reaction was performed by BioTek USA according to the schedule. The results were analyzed using SPSS statistical software version 21 and supplementary statistical methods such as t-test and ANOVA were performed. Also, $p < 0.05$ was considered as the level of significance.

Table 1: Primer sequence of genes used in the study

Amplicon Size(bp)	Sequence(5'to3')		Gene
158 bp	ACCGCAAACGCTTTATGCTG AAAGATGGCATCTGGCGGA	Forward: Reverse:	COX2
100 bp	5'-CCTCTGACGTCCATCATCTC-3' 5'-ATCTTCTCGTGCCGTCGCTT-3',	Forward: Reverse:	Her2
101 bp	GGAAGGTGAAGGTCGGAGTCA GTCATTGATGGCAACAATATCCAC	Forward: Reverse:	GAPDH

RESULTS

The aim of this study was to evaluate the effect of hydroalcoholic extract of *A.retroflexus* L. seeds on Cox2 and Her2 genes in induced colorectal cancer in an animal model of Balbc mice. Seed extraction was performed by Soxhlet method. The results showed that the yield of the prepared extract was 15.12 g. The anti-cancer activity of whole plant seed extract at concentrations (31.25, 62.5, 125, 250, 500, and 1000) µg/ml was tested against CT26 (clone) cancer lines. The cell survival percentages of each of the extracts after 3 hours are given in Figure 1. The comparison of

cell survival percentage showed that there was a significant difference between all data ($p < 0.05$).

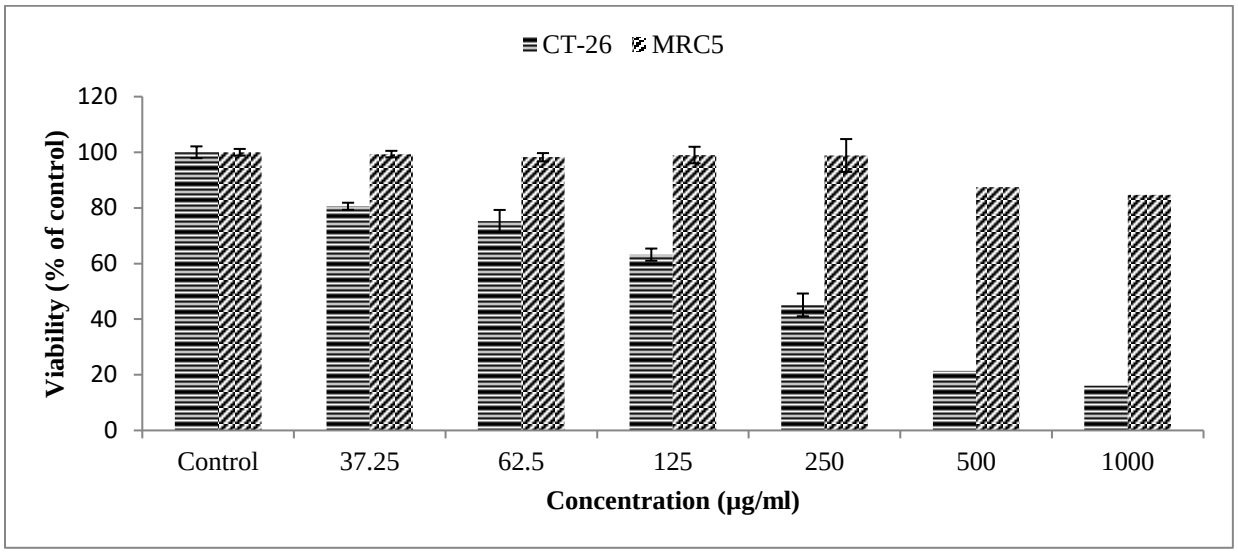

Chart 1: Effect of different concentrations of extract on CT26 cells after 48 hours by MTT method

The effect of *A. retroflexus* L. seeds extract on inhibiting the growth and death of cancer cells depends on the concentration and with increasing the concentration of the extract, the death of cancer cells increases, which is significant compared to the control sample ($p < 0.05$).

MRC5 cell line as a normal cell line against different concentrations of the extract did not show a significant reduction in survival ($p > 0.05$).

Chart 2 shows the Cox-2 and her2 genes expression in the study groups in mice. Evaluation of gene expression by real time PCR in the tumor group showed a significant increase compared to the healthy control group ($p > 0.05$). Gene expression in the treated group decreased significantly compared to the tumor group ($p > 0.05$).

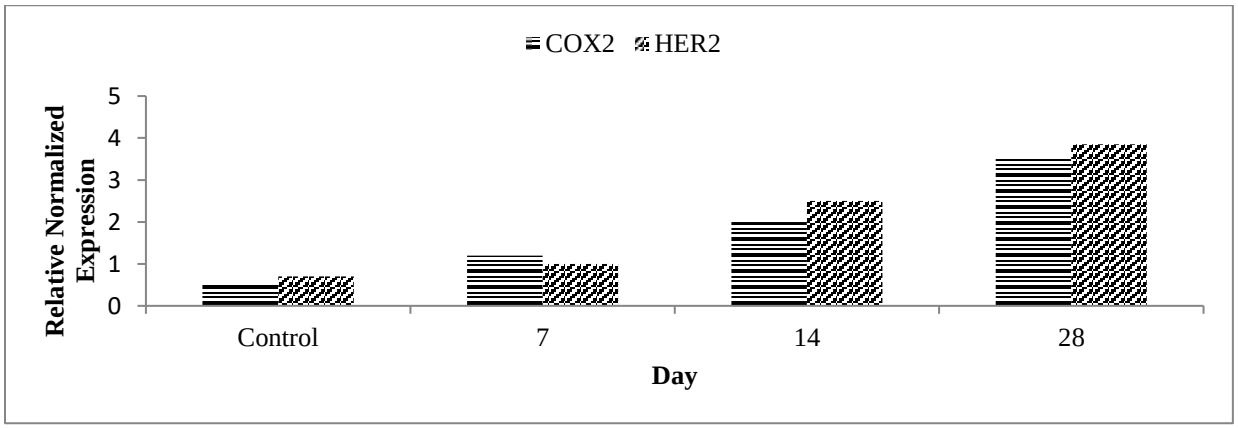

Chart 2: Cox2 and Her2 genes expression in cancer specimens without *Amaranthus retroflexus* L. seeds extract treatment. The chart columns from left to right include control sample, sample of day 7, 14, and 28

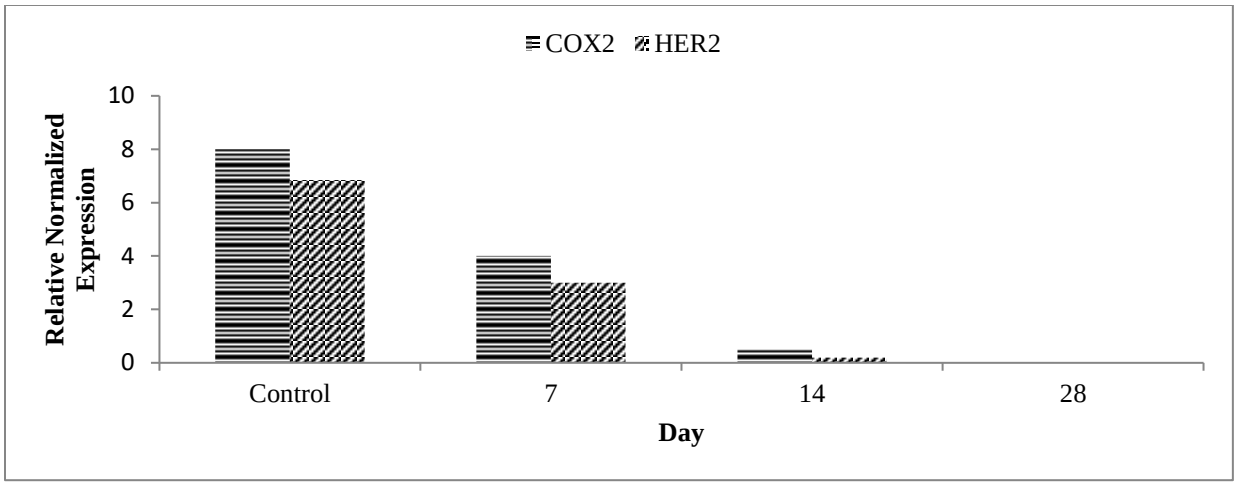

Chart 3: Cox2 and Her2 genes expression in cancer specimens treated with *Amaranthus retroflexus* L. seeds extract. The chart columns from left to right include control sample, sample of day 7, 14, and 28

DISCUSSION

Since cancer, as one of the main concerns of human society today, has brought a lot of social, economic, and psychological costs to society studies to identify compounds with anti-tumor properties and high ability to inhibit the growth of cancer cells at lower const are developing exponentially (Seidler and Huber, 2020). Due to the side effects of industrial compounds and anti-tumor chemical drugs, researchers have decided to find natural compounds with anti-tumor properties. For many centuries, herbs and herbal extracts have been used as a treatment for diseases (Khan et al., 2019). Today, many medicines are derived from herbal sources. *Amaranthus Retroflexus* L. is from the Amaranthaceae family that has four carbons, which is herbal, single-stemmed, and with an upright stem in red. The compounds in *Amaranthus Retroflexus* L. have given high antioxidant properties to this plant (Dino et al., 2017).

Among the various toxicity tests, radioisotope composition and MTT assay are the most appropriate. Mitochondria are essential organs that play an important role in cellular metabolism. MTT assay is an important method for assessing mitochondrial damage.

In recent years, attention to molecular changes, especially in the level of gene expression has increased to identify the molecular causes and factors of cancer. Important mutations in some genes have been identified as mechanisms of intestinal cancer. The cox2 and her2 genes stimulate proliferation in a variety of cancers, including clones (15). In this study, cox2 and her2 genes expression increased over time after tumor induction, which is consistent with other studies that indicate high expression of cox2 and her2 genes in colorectal malignancy.

Khelwatty et al. (2014) studied the expression of HER family members in tumor samples from 86 patients with colorectal cancer.

Dukes'c and Dukes'D tested for metastasis using immunohistochemistry (Khelwatty et al., 2014). Pham et al. (2015) showed that the relative expression of Her2 gene in tumor cells has increased relative to the normal state.

The results of this study showed that HER2 plays an important and effective role in the early diagnosis of colon cancer as a biomarker. Moreover, changes in the expression of these genes play an important role in the differentiation of intestinal cancer cells and tumor progress (Phenasi et al., 2015).

Cox2 expression is associated with cancer of clone tissue and its inhibitors show protective effects against clone cancer (Sheng et al., 2020).

Amaranthus Retroflexus L. seeds suppress the activity of these genes at both the expression surface and dimension and by preventing their expression, they inhibit the growth of many tumor models in various laboratory animals (Stratton et al., 2002).

According to the results of previous and recent studies, cox2 and her2 genes are important factors in the prognosis of clone cancer and are associated with cancer malignancy.

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