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Research Article

Ethanol Extract of Cordia myxa L Fruit Increase Expression of Antioxidant Enzymes and Tumor Suppressor PTEN Genes in MCF-7 Breast Cancer Cells

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ABSTRACT
In the current study, the cytotoxicity of an ethanol extract from the fruits of Cordia myxa was studied, in addition to the effect of the extract on the gene expression of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in human breast adenocarcinoma cells (MCF7). The study also included the effectiveness of the extract to stimulate tumor phosphatase and tensin homolog (PTEN) gene expression. The results proved that treating MCF7 cells with C. myxa ethanol extract led to a significant decrease in cell vitality (up to 70%), and the cells treated with ethanol extract showed higher expression activity of all antioxidant enzymes with real-time PCR results at a concentration of 455.8 µg / mL, in this way, it overpowered the cells exposed to H2O2 (200 µM) and not treated with the plant extract that showed reductions in the expression of enzymes SOD, GPx and CAT., we demonstrated the activity of C. myxa extract to increase PTEN expression in MCF7 cells approximately 5 to 6 times compared to cells that were not treated. Results of our study indicated that C. myxa Fruit is useful as a curative agent in prevention diseases resulting from oxidative exertion.

Keyword: antioxidant enzymes, PTEN gene, Cordia myxa,

INTRODUCTION

Free radicals, which arise in oxidation processes, are essential for producing energy to fuel biological processes in most organisms. However, the immoderate production of free radicals including superoxide, hydroxyl, peroxy, etc.[1]. Many common disorders, including, cardiovascular disease, inflammation, cancer, diabetes, and neurological diseases, which are caused by damage to fats, proteins and DNA in cells as a result of the formation of free radicals. [2]. The endogenous oxidative enzymes represented by superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) are usually incomplete to protect the organisms from free radical damage[3]. According to their source, antioxidants are classified to include enzymatic, non-enzymatic, and dietary antioxidants such as phenols, flavonoids, phenolic acids, carotenoids, vitamins and minerals. There is ample evidence that medicinal plants can be used in conventional and / or alternative modern medicine. Natural antioxidants are usually derived from plant sources, and potency is determined by plant species, variety, extraction, processing methods, and growing environment [4]. Recently, the use of herbal medicines has increased as one of the most popular areas for various therapeutic conditions [5]. Pharmacological activities, economic viability, and low toxicity, all of these have made medicinal plants a target of study. [6]. Cordia is one of plant genus which is belonging to the family Boraginaceae [7], it has been used for a long time in the various traditional systems of medicine. Plants C. dichotoma, C. latifolia, C. macleodii, C. myxa, C. rothii and C. obliqua are being used in Ayurveda, Unani and Siddha systems of medicine. Most of these species are used for treatment of wound, boils, tumor [8]. Apoptosis or programmed death of a living cell is the process by which a living cell kills itself in multicellular organisms. It can be induced by using certain stimuli, such as ionizing radiation, toxins, and anti-cancer drugs. Inducing apoptosis has been shown to be an effective and promising way to kill cancer cells [9]. Several plant extracts and phytochemical can be used to induced cancer cell lines [10]. Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene that plays an important role in many cellular processes, [11], so the loss of PTEN activity

contributes to the development of anti-cancer drugs [12]. PTEN is a passive regulator of the PI3K pathway that plays a major role in regulating the PI3K/Akt signaling pathway which is necessary to maintain the integrity of essential cellular processes, cell growth, survival, death, and metabolism. Low or ineffective PTEN leads to hyperactivity of RTK/PI3K/Akt signalling and thus cancer formation and evolution [13].

MATERIALS AND METHODS

The fruits were collected from region Abe alkasab in basrah city and authenticated by Prof. D.Abd-Ridha Al-Mayah, College of Science/ University of Basra/Iraq as Cordia myxa L. The fruits were carefully cleaned and washed with running tap water several times, its seeds removed and then airing in shade and finally crushed by an electric blender.

Preparation of the ethanol fruit extract of C. myxa

The ethanol extract was prepared by soaking 100 g of C. myxa fruits in 700 ml ethanol 95% and 300 ml distilled water, with shaking for 2 d and kept in a refrigerator. The extract was filtered. The filtrate was centrifuged at 3000 r/m for 10 min after evaporated by a rotary evaporated apparatus (Swizerland) [4] and the residue was dissolved again in DMSO 10%. Extracts were preserved at −20 ◦C until further analyses.

Cell culture

Human breast adenocarcinoma cell line (MCF7) was obtained from Baghdad Cancer Research Center. The MCF7cells were maintained in Dulbecco's Minimal Essential Medium (DMEM), low glucose (Invitrogen, Carlsbad, CA) supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin, (Invitrogen, Carlsbad, CA)at 37°C in a humidified atmosphere of 95% air and 5%CO2 as [15].

Cell proliferation assay

The influence of ethanol extract on proliferation/inhibition MCF7 was analyzed using MTT reagent using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) as [16], MCF7 monolayers were trypsinized and then the suspensions were adjusted to a concentration of 10^4 cell / ml in the growth medium. The cells were then seeded in plates 96-well at a volume of 100 µl in each well and the cells were incubated at 37 ◦C in 5% CO2 for 24 h. After 24 h, the plant extracts, at different concentrations (200, 300,400,500,600 µg/ml) were added into the plat wells and the cells were further incubated for 48 h. After 48 h the media and ethanol extract were removed. MTT staining was added in each well and 90 µl of the media free serum the plates again incubated for 3 h. After incubation, MTT is removed and 100 µl of DMSO was added instead of 20 min in the dark. Finally the absorbance is taken at 490 nm with ELISA reader and the cytotoxicity was calculated as follows: Inhibition percentage was calculated as control-test/control*100 [17] and IC50was assessment and used for the following analysis of fruit extract on antioxidant and tumor gene expression patterns in MCF7.

Antioxidants enzyme activities assay

MCF7 cells were seeded at a density of 10^4 cell / ml in the growth medium. The cells are then seeded in 96-well plates at a volume of 100 µl in each cells/well. Pre- treatment with the IC50 concentration of the plant extracts. Following the pre- treated with the extracts. The cells were treated with 200 µM of H_2O_2 after 2 h of first exposure to the plant extracts. After incubation 24 h, aspirated culture media and the cells were trypsin zed and then centrifuged at130×g for 5min for collecting. Cells were washed twice with PBS before total cells RNA was extracted [18].

Tumor suppressor gene analysis

The anticancer activity of ethanol extract of C. myxa was tested against MCF7 cells. The cells were seeds and treated as in 2.4. Barring exposed to condition of oxidative stress, the cells were incubated for 24 h, and collected for total cellular RNA extraction after culture media were aspirated.

Analyses expression gene in MCF7 cells treated with the ethanol fruit extract of C. myxa

Total cellular RNA was isolated using RNA–Spin TM Total RNA Extraction kit (iNtRON, Biotechnology, Inc), according to the manufacturer's instructions. 1µg of total RNA was reverse-transcribed used oligo(dT) primer. The extracted RNA was quantified and its quality examined using a Nanodrop ND-2000 spectrophotometer (Nanodrop). First strand cDNA synthesis was made using oligo (dT) and Super Script III Reverse Transcriptase (iNtRON, Biotechnology, Inc).

mRNA levels quantification by real-time PCR

cDNAs Using as a template, quantitative real-time PCR was performed out using the Green SYBR PCR Master Mix (RealMODTM Green SF 2X qPCR mix) in a real-time PCR, as to the manufacturer's instructions, used specific primers. Sequences of CuZnSOD, GPx, CAT, PTEN and β-actin primers that were used in Real Time PCR to evaluate gene expression in (table1). After denaturation initial (95 c° for 5 min), 40 PCR cycles were designed as following conditions: 95 c° for 5 s; 60 c° for 10 s Data were normalized to β-Actin mRNA expression as an internal reference gene. Each sample was carried out in triplicate and relative

gene expression was assessed using the comparative Ct method ($2-\Delta\Delta Ct$) [19].

Table.1. qPCR Primers Sequences

Primer	Oligonucleotide sequence	Product size(bp)	Reference
CuZnSOD	5′ACGGTGGGCCAAAGGATGAA-3′ 5′-TCATGGACCACCAGTGTGCG-3	151	[18]
GPx	5′-TCGGTGTATGCCTTCTCGGC-3′ 5′-CCGCTGCAGCTCGTTCATCT-3′	150	
CAT	5′-CCAACAGCTTTGGTGCTCCG-3′ 5′- -GGCCGGCAATGTTCTCACAC-3′	180	
PTEN	5′- AAG GCA CAA GAG GCC CTA GAT TTCT- 3′ 5′- ACT GAG GAT TGC AAG TTC CGC CA3′	148	[20]
β-actin	5′- CCTGGCACCCAGCACAAT-3′ 5′- GCCGATCCACACGGAGTACT- 3′	138	[21]

Statistical analysis

The statistical analysis was done by using statistical program SPSS (Statistical Package for Social Sciences) version 24. One-way ANOVA were used to compare between means under probability level $P \leq 0.05$

THE RESULTS

In the present study, ability of C. myxa fruits ethanol extract to induce antioxidant enzymes SOD1, GPx and CAT an addition to its effect on the expression level of PTEN gene in MCF-7 cell was assayed. The essential step in evaluation plant extract suitabile for further purposes is evaluation its cytotoxicity [22]. The cytotoxic effectiveness of ethanol extract of C. myxa fruits at 200–600 µg/mL concentrations was investigation in MCF-7 cells line using cell viability MTT assay, after 28 h of incubation with C. myxa extract significantly decreased with the increased of extracts concentration (figure1), The concentration of extract that caused a 50% reduction in cells viability (IC50) was 455.8 μg/ml. The highest concentration tested was 600 μg/ml, reduced cell viability to 70%. The IC50 concentration was subsequently used to treat the cells for the gene expression analyses.

Gene expression analyses in MCF7 cells treated with the ethanol fruit extract of C. myxa

There are many studies confirming that several antioxidants working as cellular signals that control the antioxidant enzymes level [23].

Evaluate the effects of ethanol fruits extract of C. myxa on the antioxidant enzymes expression, inclusive CuZnSOD, GPx and CAT, in MCF-7 cells dealings with H2O2. SOD1, GPx, and CAT gene response was investigated by quantitative real-time PCR in MCF7 cells by treated with IC50 concentration plant extract for 24 h. The induction of MCF7 cells treatment with H2O2 (200 μM) decreased the expressions of SOD1, GPx, and CAT enzymes, while all the antioxidant enzyme activities in the cells were significantly enhanced in the cells after pre-treatment with fruit extract compared with treated and untreated cells induced by H2O2 (figure2) . Data were analyzed by $\Delta\Delta$ CTs and normalized to (β actin) house-keeping gene. The most important defenses in cells are antioxidant enzymes due to they regulate the redox state in cells by eliminating excess free radicals [24]. Testimony from previous studies confirms that superoxide dismutase; peroxidase and lactase enzymes are involved in modulating H2O2 and is an important mediator in apoptosis. The expression of antioxidant enzymes activity by C. myxa fruits, it is attributed to the presence of biologically active metabolites. Similar to our result, [18] it was mentioned that an ethanol extract from hemp seed significantly affected the expression of antioxidant enzymes gene in HepG2 cells. Thus it appears that the expression of antioxidant enzymes is essential for inhibition of many diseases caused by free radicals such as cancer, joint stiffness and chronic inflammation [25].

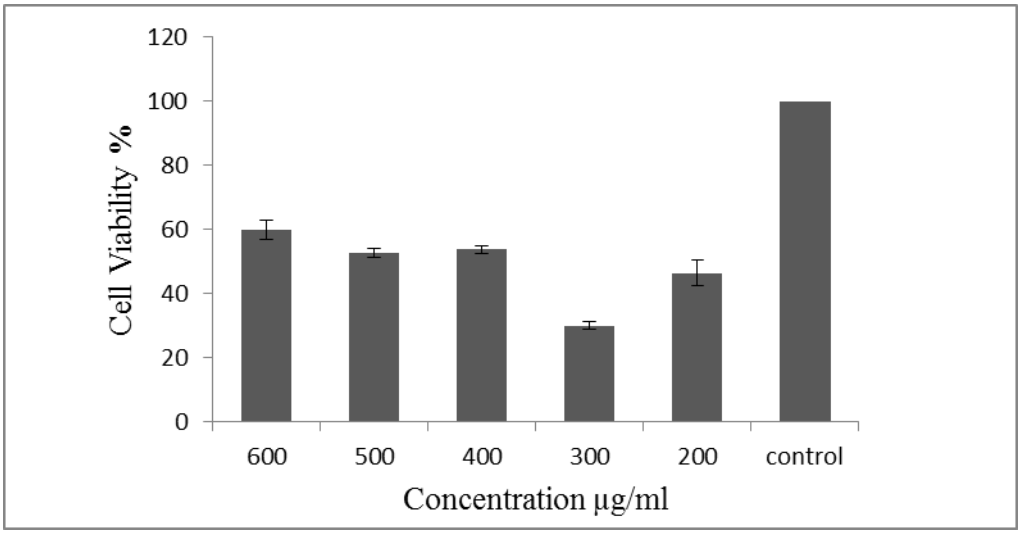

Fig.1:Effect of ethanol extract of *C. myxa* fruit on the growth of breast cancer cell line. MCF-7 cells were treated with different concentrations of extract for 48h, and the viability was assessed by MTT assay. Data are represented as means standard deviations (n = 3).

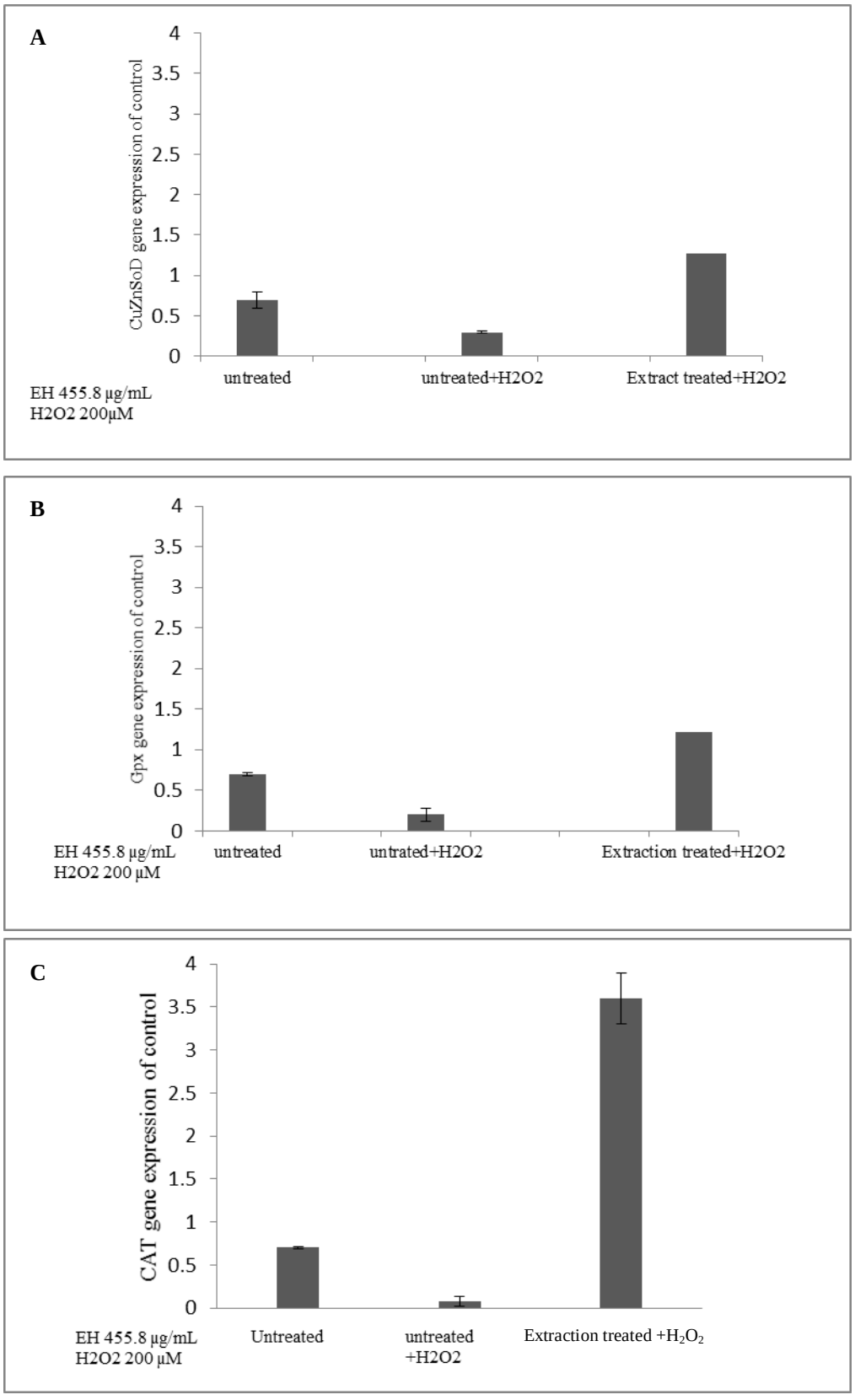

Fig.2:Effect of ethanol extract of *C. myxa* fruit on A. CuZnSOD, B. GPx, C.CAT, enzymes gene expression in unstressed and H2O2stimulated MCF-7 cells were treated with extracts for 24 h. Values are mean of three replicate determinations (n=3) ± standard deviation.

Expression PTEN gene in MCF-7 cells

The expression of PTEN mRNA was estimated in both treated and untreated MCF-7 cells. MCF-7 cells treatment with IC50 concentration plant extract showed an increase in expression of the PTEN gene approximately 5 and 6 times greater than cells untreated with the extract (Figure3). This finding is in agreement with the ability of ethanol extract to decrease the cell viabilities. Mutation in PTEN gene increased Akt activity in many tumor types [13]. PTEN acts as a regulatory control on the PI3K pathway and action, including protein kinase B / Akt stimulation, survival, proliferation, and cell migration, [26]. Therefore, a drug development strategy targeted inhibition of the PI3K / Akt pathway [27]. Whereas, some food phytochemicals have been shown to regulate the PI3K / Akt pathway and induce apoptosis, so they have been tried as anti-cancer drugs [28]. Others reported also were reached the same conclusion [29]. This monitoring indicates that the change in the PTEN gene is very important for tumor initiation and may mutually aid in other genetic modifications, so PTEN regulation has been invoked as a promising research object for cancer treatment [30].

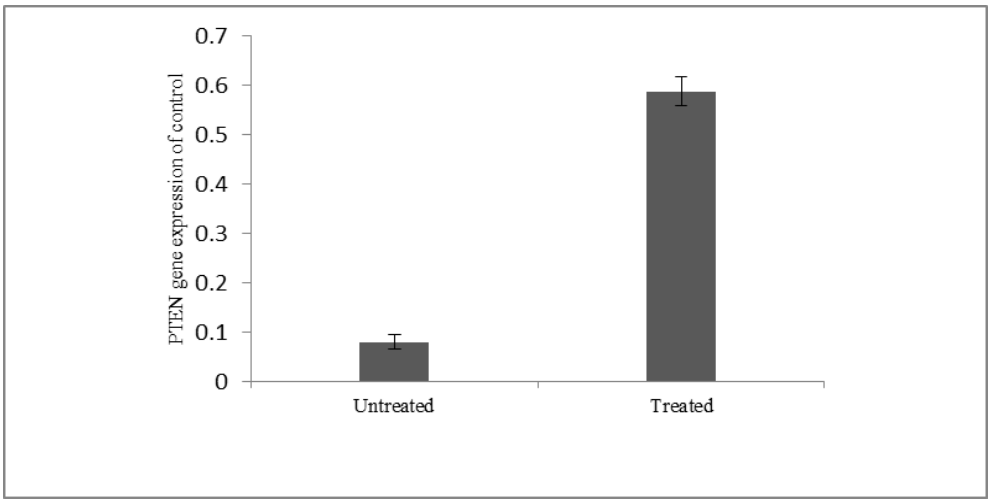

Fig.3:Effect of ethanol extract of *C. myxa* fruit on PTEN protein gene expression in MCF-7 cells. Cells were treated with IC50 concentration (455.8 µg/ml). Values are mean of three replicate determinations (n=3) ± standard deviation

CONCLUSION

This study showed the gene expression of SOD, GPx and CAT enzymes can be regulated with C. myxa fruit extract thus reducing the levels of rhizomes. This plant extract can also be used to stimulate the expression of the PTEN gene and may promote apoptosis through the ''PTEN / PI3K / AKT'' signaling pathway. Further studies needful to supply evidence of the safety and efficacy of C. myxa in treatment breast cancer.

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