RESEARCH ARTICLE

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Apoptotic Effects of *Linum album* Extracts on AGS Human Gastric Adenocarcinoma Cells and *ZNF703* Oncogene Expression

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Abstract

Introduction: *Linum album* is a medicinal plant endemic in Iran that is very important pharmaceutically. The present study concerns the effect of different extracts of *L. album* on *ZNF703* gene expression and apoptosis in human gastric carcinoma AGS cells. **Method and material:** Hydro alchoholic *L. album* extracts from various plant sources were produced by Maceration. AGS cells were treated with different concentrations (200, 400, 600, 800 and 1000 µg/ml) and the cytotoxicity potency was assessed after 24 h by MTT assay. Then, quantitative real time PCR was conducted for *ZNF703* gene expression in AGS cells. Also, cell apoptosis/necrosis was assessed with the aid of Annexin V/PI staining and quantification by flow cytometry. **Results:** *L. album* extracts exerted dose-dependent toxicity in the AGS cell line. The mRNA levels of *ZNF703* gene expression were significantly decreased with rhizome, fruit at fruiting, leaf and stem at anthesis ($P<0.001$), and leaf and stem at fruiting extracts as compared to the controls ($P<0.01$). Also, the number of apoptotic cells was increased from 2.70% (statistically significant; $p<0.05$) in untreated AGS cells to 44%, following treatment with the leaf and stem at anthesis example. **Discussion:** Our findings revealed that the *L. album* extracts can induce apoptosis and might modulate cytotoxicity by down regulating *ZNF703* gene expression in AGS cells. Therefore, this extract could be a good candidate for inhibiting cancer cell growth, especially that of gastric cancer. In addition, *ZNF703* may have potential as a therapeutic target.

Keywords: *Linum album*- gastric cancer- *ZNF703*- apoptosis

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Introduction

Gastric cancer is the second leading cause of cancer death and is usually higher in men (Ferlay et al., 2008; Forman et al., 2006; Plummer et al., 2015). To date, various methods are used to treat cancer, but response to treatment has unfortunately been very poor in most cases and associated with undesirable side effects. Thus, research to produce more efficient and less toxic drugs is essential. Various synthetic and natural compounds have been used in cancer chemotherapy (Kamangar et al., 2006; Long et al., 2010; Raskob et al., 2018). However, herbal compounds have long been used in traditional medicine and currently in chemotherapy (Gabr et al., 2018).

Many plant compounds have been identified as having anticancer effects in modern pharmaceutical science (Srivastava et al., 2005; Newman et al., 2016). Linum is a genus in the family Linaceae that includes 230 species (Talebi et al., 2012). *Linum album* is a medicinal plant that has been used in traditional medicine to treat diseases such as gastric ulcer, kidney stones, hepatitis and cancer (Xiong et al., 2011). It contains podophyllotoxin, which is a precursor of the anticancer drugs Etopside, Teniposide and Etophose (Arro et al., 2002). Among its pharmaceutical applications and physiological properties, the anti-viral and anti-tumor properties are the most important pharmacologically (Esfandiari et al., 2018).

Substantial attention has been focused on gene expression studies in tumor cells and as a pathway for inhibition of cancer proliferation. The over-expression of the *ZNF703* oncogene has been identified in cancers such as those of the breast and stomach (Yang et al., 2014). The identification of compounds that can down-regulate this gene may help us to inhibit the growth of gastric cancer.

In general, *L. album* appears to have pharmaceutical

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benefits and offer anti-cancer action to be applied to gastric cancer treatment. The present study aimed to investigate the apoptotic effect of different extracts of *L. album* on the human gastric adenocarcinoma cell line.

Materials and Methods

Preparation of extracts

Two periods of anthesis and fruiting stage of *L. album* was collected from Sohanak area, Tehran, Iran. They were then completely dried and prepared powder by electric mill was maintained into glass containers. Maceration method was used to produce the hydro alcoholic extract. 200 grams of milled powder was concentrated with alcohol (70% ethanol and water) and shaking under vacuum condition at 45°C. Extracts were then filtered using 0.45 µm filters (Millipore Inc., Bedford, Massachusetts) and divided into sterile microtubes and stored at -80°C.

In Vitro cytotoxicity assay

Cell line and culture medium

The AGS gastric adenocarcinoma cell line (NCBIC131) was purchased from the cell bank of Pasteur Institute of Iran. The cells were grown in the RPMI-1640 medium (Biosera, USA) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS) (Gibco, Netherland) and penicillin/streptomycin at a final concentration of 100 units per ml. To provide growth conditions, the cells were incubated in humidified atmosphere with 5% CO_2 at 37°C.

MTT assay

To evaluate the effect of *L. album* extracts against AGS cell survival, MTT 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide assay was performed. Briefly, about 1×10^4 cells were added into 96 well microplates and incubated for 24 h at 37°C under 5% CO_2. The concentrations of 200, 400, 600, 800 and 1,000 µg/ml of the each extracts were separately added into wells. After 24 h, 25 µl of MTT dye solution was added into each microplate well and incubation continued for 4 hours. Then, the supernatant was removed and 100 µl DMSO was added into each well to dissolve formazan crystals. After pipetting, the absorbance was measured at 570 nm using an ELISA reader. The 50% inhibition (IC_{50}) of cells was measured by utilizing the following formula:

$$(\%)\ \text{inhibition} = \frac{\text{Abs}\ 6\ \text{Control} - \text{Abs}\ 6\ \text{Test}}{\text{Abs}\ 6\ \text{Control}} \times 100$$

ZNF703 gene expression analysis

RNA extraction protocol was processed according to Cinna Pure RNA Purification Kit instruction. The quantity and quality of extracted RNA was determined using Nanodrop and gel electrophoresis. RNAs extracted from AGS cells either untreated (control cells) or treated with various concentrations of the extracts were applied to cDNA synthesis according to Revert aid First Strand Synthesis Kit instruments (Fermentas, USA). The β-actin gene was also considered as internal reference gene. All amplifications were done using Exicycler™ 96 Real-Time PCR (Bioneer, Korea). The final volume of 20 µl was consist of 2 µl $MgCl_2$ (50mmol), 2 µl 10X buffer, 0.5 µl dNTP (50mmol), 0.5 µl from each primer (10µmol), 0.4 µl from each probe (10 µmol), 0.2 µl Taq Polymerase (5u/ µl), 2 µl cDNA and 9.2 µl DEPC treated water. The qPCR program started with one cycle reaction at 95°C for 1 min, 40 cycles of 95°C for 20 s, 57°C for 40 s, 72°C for 40 s, and then final extension at 72°C for 5 min. The sequence of primers and probes are shown at Table 1.

Flowcytometric analysis of apoptosis/necrosis

The apoptosis and necrotic AGS cells were quantified using flow ctyometry method. After incubation with *L. album* extracts in six well plates for 24h, the AGS cells were harvested with trypsin treatment and following centrifugation, washed with PBS, stained by 10 µL Annexin-V- FLUOS and 5µL propidium iodide (PI) according to the manufacture's protocol (Roch, Germany). Finally, the numbers of apoptotic and/or necrotic cells were measured by flow cytometry using PAS machine (Partec, Germany). Each experiment was carried out triplicate.

Statistical analysis

The standard error of means was computed and analysis of variance (ANOVA and Tukey's tests) completed via Graph Pad Prism 5.0 software version 6.0. P value less than 0.05 was considered as the significant level. Real time PCR analysis was performed with the REST 2009 and expression fold change was determined by $2^{-\Delta\Delta ct}$.

Results

MTT assay

The cytotoxic effects of extract in different concentrations on the cancer AGS cell line are presented in Figures 1-3. After incubation for 24 h, AGS cells treated with extracts of rhizome during fruiting period showed that fruiting extract in concentration of 1000 µg/ml had maximum inhibition of cell proliferation ($P<0.001$), whereas the all of three extracts at the concentration of 200µg /ml showed no statistically significant differences relative to the control group ($P=0.99$) (Figure 1).

Treated cells with leaf and stems at anthesis and fruiting stages and flower at anthesis period revealed the same results obtained with the rhizome extracts, so that the concentrations of 1,000 µg/ml had maximum inhibition property ($P<0.01$) and concentrations of 200µg/ml did not have effect on cells' survival ($P>0.05$). AGS cells treated with the fruit extract at the fruiting stage after 24 hours showed that concentration of 1000 µg/ml had the highest and concentration of 200µg/ml had the minimum lethal effect (Figure 2 and 3). Results for survival when compared with control samples are presented as mean ± SD.

Relative quantification analysis for ZNF703 gene expression

After determining IC50 and appropriate concentrations of the extracts (Table 2), the relative transcript abundance of the *ZNF703* gene in absence or present of *L. album* extracts were measured by quantitative real time PCR (Figure 4). Our finding reveal that mRNA

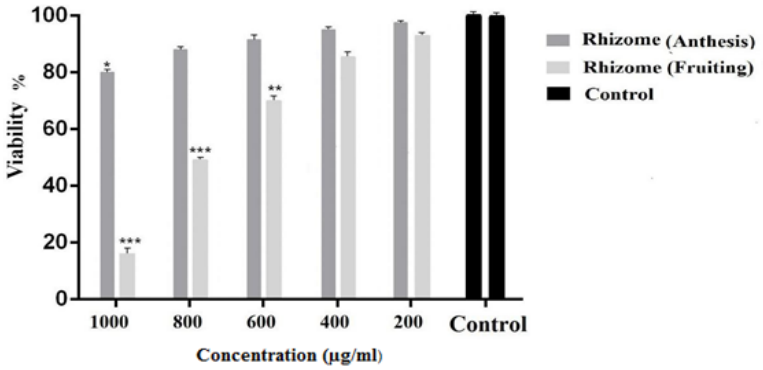

Figure 1. AGS Cell Survival after 24h Treating with Different Concentrations of Rhizome Extracts at Different Stages. Cell viability was determined by MTT assay. Absorption was measured by ELISA reader at 570 nm (*, P<0.05; **, P<0.01; ***, P<0.001).

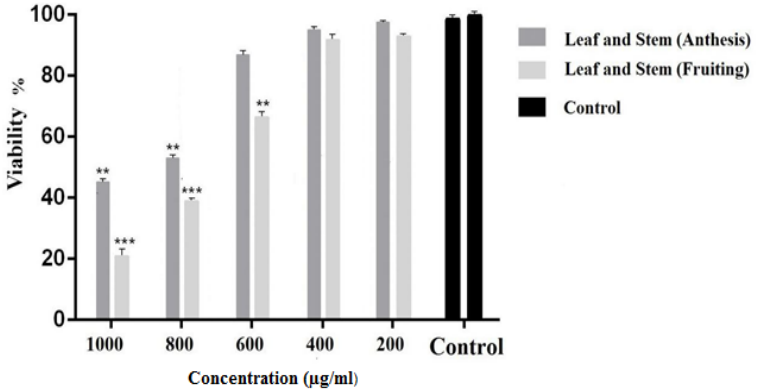

Figure 2. AGS Cells Treated with Different Extracts of the Leaves and Stems at Flowering and Fruiting Stage and Flower at Flowering Stage. Cell viability was determined by MTT assay. Absorption was measured by ELISA reader at 570 nm (*, P<0.05; **, P<0.01; ***, P<0.001).

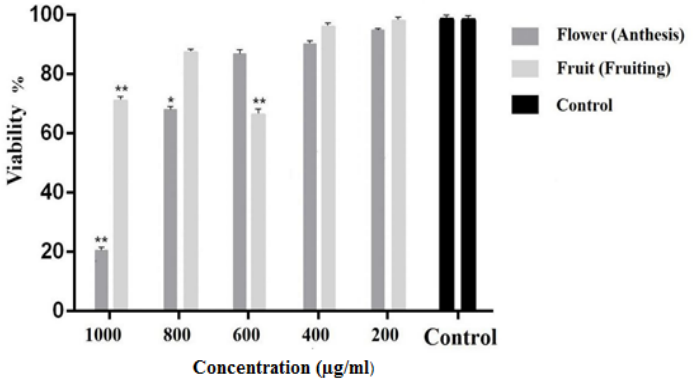

Figure 3. Cell Survival Rate after 24 Hours Treatment with Different Concentrations of *Linum album* L. fruit Extract. Cell viability was determined by MTT assay. Absorption was measured by ELISA reader at 540 nm (*, P<0.05; **, P<0.01; ***, P<0.001).

Table 1. Primers and Probes Sequences, Product Size and Most Appropriate Tm for Amplifying Target Region. Specificity and Secondery Structures of Primers and Probes were Checked by NCBI Primer BLAST and OLIGO 7 Software

Gene/Probe	Primer sequence	Tm	Product size
ZNF703 (NC_000008.11)	F:AGGGTCCTGAAGATGCTGA R: TCTTGGCGTCCAGCTCAATG	57	150
β-actin (NC_000007.14)	F: TCCTCCTGAGCGCAAGTAC R:CCTGCTTGCTGATCCACATCT	57	155
Probe	*ZNF703*:ACGGGAGTGGAGGACAGCGG β-actin: TGGAAGGTGGACAGCGAGGC	68	

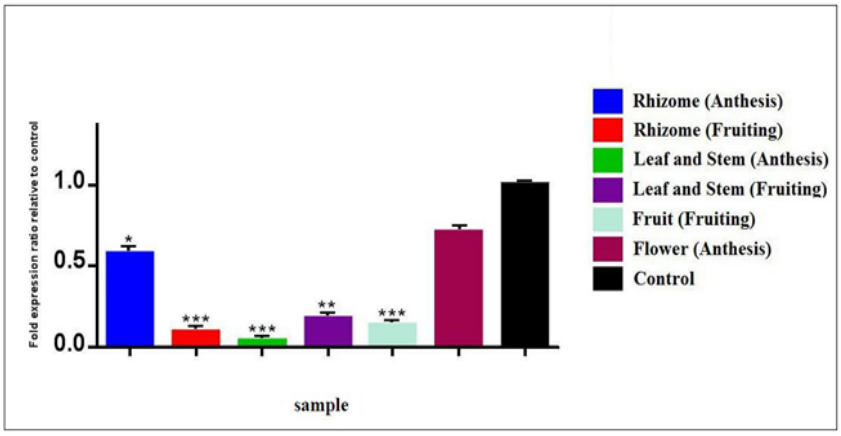

Figure 4. Expression Ratio of ZNF703 to β-actin in Treated AGS Cells than Untreated Cells. A, AGS cell lines treated with rhizome extracts; B, AGS cell lines treated with extracts of leaf and stem at anthesis and fruiting stage and flower at anthesis stage; C, AGS cell lines treated with extracts of fruit at fruiting stage. Generally, the results are indicative that Linum album extracts can significantly decrease ZNF703 expression. The effect of fruit extract is least than other extracts (*, P<0.05; **, P<0.01; ***, P<0.001).

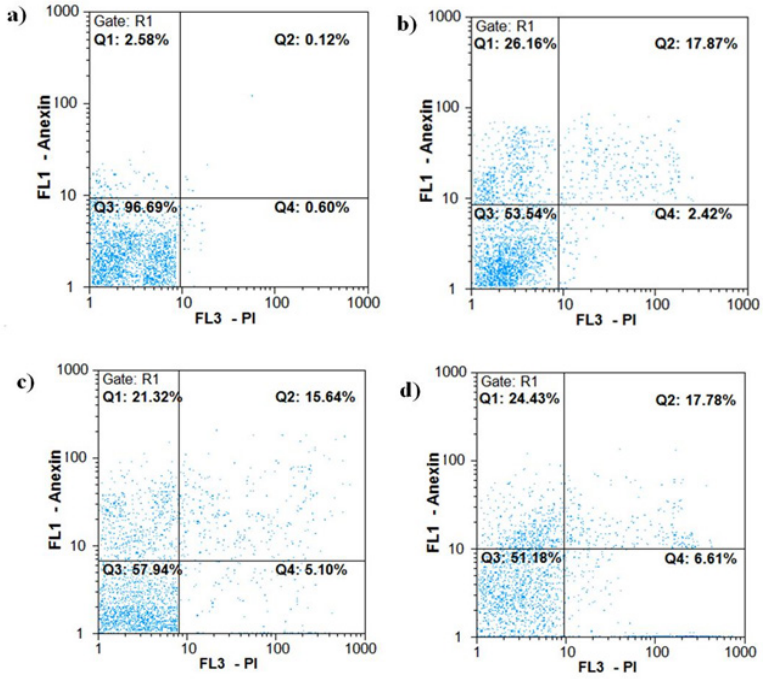

Figure 5. Flow Cytometric Analysis by Annexin V-FLUOS (FL1) in x-axis and PI (FL3) in y-axis Double Staining of AGS Cells Treated with Linum album Extracts at 24 h. Dot plots of annexin V/PI staining are shown in (a) untreated AGS cells. (b) AGS cells treated with leaf and stem at anthesis showed 17.87% early stage apoptosis and 26.16% late stage apoptosis. (c) AGS cells treated with fruit at fruiting had 15.64%early stage apoptosis and 21.32% late stage apoptosis. (d) AGS cells treated with rhizome at fruiting exhibited 17.78% early stage apoptosis, 24.43% late stage apoptosis.

level of *ZNF703* were significantly decreased in the AGS cells with rhizome, fruit at fruiting stage, leaf and stem at fruiting period (P<0.001), and leaf and stem at fruiting treatments compared to the controls (P<0.01). The expression of *ZNF703* transcripts in cells cultured with flower at anthesis did not differ from control cells.

Table 2. IC_{50} of *Linum album* Extracts at Different Stages

Extract	IC_{50}
Flower(Anthesis)	880 ± 0.48 µg/ml
Rhizome (Anthesis)	350 ± 0.78 µg/ml
Rhizome (Fruiting)	705 ± 0.56 µg/ml
Leaf and Stem (Anthesis)	802 ± 0.37 µg/ml
Leaf and Stem (Fruiting)	690 ± 0.82 µg/ml
Fruit(Fruiting)	894 ± 0.42 µg/ml

In vitro Apoptosis/necrosis assay

To further determine apoptosis induction of *L. album* extracts, AGS cells were stained with Annexin-V/PI assay, followed by flow cytometry. The characteristic result of flow cytometry was depicted in Figure 5. The upper right quadrant represents the percentage of early apoptotic cells and the upper left quadrant depicts

the percentage of late apoptotic cells. The fully apoptotic cells are those in the upper right and left quadrants. The number of apoptotic cells was increased from 2.70% (statistically significant; $p<0.05$) in untreated AGS cells to 44 %, 36.96%, 42.21% following treatment with leaf and stem at anthesis, fruit at fruiting , and rhizome at fruiting of *L. album*.

Discussion

Cancer is a major health problem in society (Ward et al., 2014; Jemal et al 2010). Determining the therapeutic effects of natural compounds that are effective against cancer cells is a key priority. Gastric cancer is often asymptomatic or has nonspecific symptoms in the early stages of the disease, which is one reason for its relatively poor prognosis (Orditura, et al., 2014). Natural compounds offer advantages over synthetic drugs, including fewer side effects, so medicinal herbs have long captured the attention of researchers. In the present study, the in vitro anti-proliferation effect of different extracts of *L. album* on human gastric adenocarcinoma AGS cells was assayed. Additionally, the role of these extracts in suppressing of *ZNF703* gene expression was evaluated.

Compared with other extracts, rhizomes in the anthesis stage showed an inhibitory effect on the growth and proliferation of cancer cells at the highest concentration. All extracts had no significant effect on cell viability at a concentration of 200 µg/ml, whereas the concentration of 1,000 µg/ml was the most effective. Fruit extract was the weakest anti-proliferative extract.

Fruit extract was the weakest anti-proliferative extract. Several studies have evaluated the anti-proliferative effect of herbal extracts on cancer cells, but none were found that have evaluated the scavenging effect of *L. album* against the gastric cancer cell lines. Amirghofran et al. studied the anti-cancer effect of *L. persicum* and *Euphorbia cheiradenia* on leukemia cell lines. They found that both herbs have cytotoxic effects through the induction of programmed cell death (Lalaleo, et al., 2013). Evaluation of the anti-growth properties of methanol extract from the Croton species on ten human cell lines showed that *C. erythroxyloides* leaf extract is the most powerful for inhibition of prostate and ovarian cancer cell lines (Savietto, et al., 2013). Sadeghi et al. showed that podophyllotoxin, the most important compound found in *L. album*, promotes apoptosis by suppressing *TuBB3* and *TOPIIA* expression in bladder carcinoma and leukemia cell lines. This suggests consideration of this compound as a potential anti-cancer agent (Sadeghi, et al., 2015).

Genome-wide analysis and next-generation studies of cancer have revealed that 8p12 amplification occurs most often in breast cancer. One of genes located in this region is *ZNF703*. It acts as a transcriptional repressor and shows oncogenic activity that affects various aspects of breast cancer (Yang et al., 2017). Holland et al., (2011) reported that 8p12 amplification occurred in 13% of luminal B breast cancer. Reynisdottir et al., (2013) found that *ZNF703* mRNA over-expression was significant in ER-positives compared to ER-negatives and this correlates with DNA copy number of *ZNF703*, suggesting the gene as a driver of cancer pathogenesis. A few studies have examined the role of *ZNF703* in gastric cancer. It seems to be involved in the tumor pathogenesis. Gongli Yang et al., (2014) analyzed *ZNF703* gene expression and its functions in gastric carcinoma. Their IHC results showed that *ZNF703* was up-regulated in invasive tumors and was related with poor clinical outcomes. Functional studies on RNA interference have revealed that *ZNF703* acts as a oncogene in gastric cancer. Our results alongside of mentioned findings regard to *ZNF703* amplification, over expression and function are indicating that the gene play an important role in development of cancer, therefore its inhibition and down regulation can be a promising approach in combat with cancers especially gastric cancer. Our quantitative PCR results also disclosed that extracts of *L. album* are able to reduce significantly of *ZNF703* gene expression.

The results of the current study alongside these findings relating to *ZNF703* amplification, over-expression and function indicate that the gene plays an important role in the development of cancer. Therefore, its inhibition and down-regulation can be a promising approach to combating cancer, especially gastric cancer. The quantitative PCR results also reveal that extracts of *L. album* are able to significantly reduce *ZNF703* gene expression.

Further investigation included the annexin V/PI assay, followed by flow cytometry. In the early apoptosis stage, phosphatidyl serins (PS) in the membrane translocated from the inner leaflet to the outer leaflet of the cell membrane. Annexin–V with PI staining could be used for staining to disclose PS by flow cytometry. The annexin V^+/PI^- cells identified the early stage of apoptosis and annexin V^+/PI^+ cells identified the late stage of apoptosis. (Nie, et al., 2014). The staining results showed many annexin V^+/PI^+ cells in the cells treated with leaf and stem extract at the anthesis stage as compared to the untreated AGS cell line.

Our results reveal that AGS cells have an apoptosis effect when they are treated with *L. album* extract and that the cytotoxic effect of *L. album* on AGS cells was induced by apoptosis rather than necrosis. The apoptotic properties of extracts used in the current study may be due to presence of PTOX in *L. album*. PTOX is a mitotic spindle inhibitor with pharmaceutical applications as an anti-malarial and anti-fungal and as an inhibitor of the immune system in organ transplants (Esfandiari, et al., 2018). It is proposed that a PTOX-rich rhizome, rather than fruit, is the key player in creating the difference in anti-growth rates.

Briefly, our findings clarified that methanol extracts of *L. album* have the potential to be used as anti-tumor vehicle. Also, *ZNF703* seems to have determining role in tumor formation and proliferation that it's suppressing may be the mechanism of anti-proliferatory activity of the extracts used in this study. Therefore, our findings highlighted that *L. album* extract can be consider as an anti-cancer medicine and *ZNF703* should be a good drug target in the way of gastric cancer treatment. More investigations about side effect of *L. album* extracts, applying them in

in vivo and evaluating the effect of different extracts on the genes involved in cancer, especially stomach cancer are suggested to use of this product for patient and identifying new molecular targets.

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