Effects of *Alkanna bracteosa* extract on the expression level of HSP90α and HER2 genes in human gastric cancer cell line

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**Abstract**

Gastric cancer was classified as the third most deadly cancer among all other cancer types. The HSP90 and HER2 genes play essential roles in the stability and function of high-expression proteins that cause malignancy. The aim of this research was to investigate the influence of the alcoholic *Alkanna bracteosa* extract on the expression of HSP90α and HER2 genes in AGS cell line. Therefore, the methanolic extraction was isolated from aerial parts of the plant and AGS and HuGu cell lines were analyzed using 102.4–0.05 mg ml⁻¹ dose concentrations in serial dilution; to measure the cell toxicity by MTT assay. Furthermore, real-time PCR analysis measured the expression level of HSP90α and HER2 genes using the IC50 dose concentrations. Quantification of apoptosis was analyzed by Annexin/PI kit in flow cytometry and DNA fragmentation tests. The results of MTT assay represented the IC50 dose concentration of 0.8 and 3.2 mg ml⁻¹ for AGS and HuGu respectively. The rate of HER2 gene expression was significantly decreased in AGS cells treated with 0.8 mg ml⁻¹ dose concentration compared to control. The exposure of AGS treated cells with 0.8 mg ml⁻¹ dose concentration after 24 h represented 24.3% apoptosis and 13.3% necrosis. The agarose gel represented the DNA fragmentation pattern of apoptosis. This study demonstrated the significant differences between the cell viability rate, gene expression level, and apoptosis of the *Alkanna bracteosa* extract on AGS cells. These results demonstrated the first report of which the *Alkanna bracteosa* would be an effective candidate for possible treatment of Gastric cancer.

**Keywords** Gastric cancer · *Alkanna bracteosa* · Apoptosis · HSP90 and HER2 genes · Cell cytotoxicity

**Introduction**

Cancer is a set of different abnormalities, which can be defined as the accumulation of genetic and epigenetic factors in cellular pathways which regulating cell proliferation, differentiation, and death mechanisms. The cancer cell deficiencies categorized into two types which including loss-of-function alterations affecting tumor genes and gain-of-function alterations affecting oncogenes (Teh & Fearon 2020). The cancer treatment modalities which are reported, including surgery, radiation therapy, and systemic treatment, as well as chemotherapy, targeted therapy, hormonal therapy, and immunotherapy (Abuei et al. 2019; Miller et al. 2019; Pirouzfar et al. 2020). Although radio- and chemotherapy are the most common and non-invasive cancer treatment methods, it can cause serious damage to the normal cells (Mortezaee et al. 2019). The fifth most recognized cancer and the third leading cause of cancer death were dedicated to gastric cancer (Bray et al. 2018). Surgery combined with chemotherapy and radiotherapy was introduced as a practical method for gastric cancer treatment (Sitarz et al. 2018) and many studies are still underway to develop these methods.

Proto-oncogene mutations may turn these genes into oncogenes, which may lead to extreme cell division and, ultimately, turn the normal cells into cancerous cells (Chowdhury 2019). The ErbB receptor family consists of 4 members, (i) ErbB1 (EGFR, HER1), (ii) ErbB2 (HER2,
with gastric cancer (Gravalos & Jimeno 2008). The level of ErbB2 is reported in 7–34% of patients involved
in many biological and biochemical processes such as anti-inflammatory, anti-tumor, anti-microbial, and anti-coagulant
mechanisms (Papageorgiou 1980; Papageorgiou et al. 1999).

The main purpose of this study was to estimate the significant impact of the methanolic Alkanna bracteosa extract
on the expression level of HSP90α and HER2 genes in AGS and HuGu cell lines and to measure its apoptotic effects to
predict its toxic level on the cancer cells. Given that gastric cancer is a disease with complex and exhausting treatment
process, proposing a way to treat and cure of this disease would be a valuable achievement to introduce to medical
science.

Materials and methods
Plant preparation and extraction
Aerial part of dried, powdered Alkanna bracteosa plant was obtained from the plant bank of the Iranian biological
resource center (IBRC), Tehran, Iran. After that, dried powdered Alkanna bracteosa plant (10 g) were soaked with
80% methanol (Merck, Germany) at 40°C for 24 h. To continue, the alcoholic extracts were concentrated using a rotary
evaporator (40°C) and the dried extracts were preserved in 2–8°C for subsequent experiments.

Cell culture preparation
Human Gastric Adenocarcinoma cell, known as AGS, and Human Gum fibroblast cell, known as HuGu, were obtained
from the human and animal cell bank of the IBRC, Tehran, Iran. AGS and HuGu cell lines were cultured in DMEM: F12
(1:1 Vol/Vol, Gibco, USA), medium supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, USA) and

Alkanna is classified as a plant, which is a sort of the Boraginaceae family and includes about 50 various species. It can grow widely in a variety of areas which including Europe, Mediterranean local places, and western Asia. A variety of enantiomeric hydroxyl naphthoquinone red pigments which including alkanin, shikonin, and their derivatives are found among Alkanna species (Assimopoulou et al. 2006). Alkanna bracteosa, a subfamily of Alkanna, is a popular and natively adapted species in Iran (Salimikia et al. 2015). Naphthoquinones derivatives including alkanin and shikonin represent effective interferences in many biological and biochemical processes such as anti-inflammatory, anti-tumor, anti-microbial, and anti-coagulant mechanisms (Papageorgiou 1980; Papageorgiou et al. 1999).

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2 mM L-Glutamine (Sigma, USA). The HuGu cell line was further treated with 1% non-essential amino acid supplement (MEM) and then were kept in a humidified incubator at 37°C containing 5% CO₂.

**Investigation of cell viability by MTT assay**

The cell survival rate was measured by MTT assay ([3-(4, 5-dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide]) (Sigma, USA) after the exposure of the cells to the *Alkanna bracteosa* methanolic extract. In this regards, the cell lines were seeded in 96-well culture plates at about 10⁵ cell density/well for AGS and HuGu cell lines. The cells were then incubated according to the previous cell culture condition. After 24 h, the cell culture medium was replaced with fresh medium containing different concentrations of the *Alkanna bracteosa* extract to be tested. The applied dose concentrations for AGS and HuGu cell lines were included 102.4, 51.2, 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05 mg ml⁻¹. The treated cells were then kept in incubator for further 24 and 48 h. After that, the culture medium was replaced with fresh medium containing MTT powder with a final concentration of 0.5 mg ml⁻¹. Finally, the cells were incubated for an extra 4 h and the medium containing MTT solution was removed as well as the created formazan crystals were dissolved in 100 μl dimethyl sulphoxide (DMSO) (Sigma, USA). The absorbance was recorded with a microplate reader spectrophotometer (Biotek ELX800, USA) with 570 nm absorbance filter and 630 nm reference filter. Negative and positive control was considered as the untreated cells and treated with 10% DMSO respectively. The cytotoxic effects of the compounds were determined according to the average absorbance rate of each treated group divided on the average absorbance of negative control group and reported as the percent of cell viability. The SPSS ver 22 program graphed the viability percent versus dose concentration curves, and the IC50 values were assessed (Mousavi et al. 2018).

**Real-time quantitative PCR of HER2 and HSP90α gene expression**

The 10⁶ density of the AGS and HuGu cells were then cultured and treated using *Alkanna bracteosa* plant extract. After 24 h, RNX-Plus kit (Sinaclon, Iran) was used to extract the total RNA from the cultured cells. For quantitative real time polymerase chain reaction (qRT-PCR), 2 μg of total RNA was used for the reverse transcription reaction and cDNA synthesis by using a BIOFACT 2X RT Premix kit (Biofact, South Korea). qRT-PCR analysis was performed with SYBR-Green Real-Time Master Mix (Biofact, South Korea) and using by the Step one plus ABI system (Applied bio systems, USA). The primers sequences used for qRT-PCR were listed in Table 1 and designed to include at least one intron on the corresponding genomic DNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The amplification process was performed under the following conditions which including 1.5 min at 95°C for one cycle, 20 s at 95°C for 40 cycles, and 40 s at 60°C for one cycle. The final extension cycle was done in 30 s at 72°C. All PCR products were performed in triplicate and the standard curves for all the products were plotted and reported (Tavakolian et al. 2019).

**Hoechst DNA staining**

AGS and HuGu cells were cultured in 35 mm² cell culture plates covered with a pre-cleaned 18 × 18 mm coverslip glass. In the next day, the cell attached and the culture medium was replaced with fresh medium containing *Alkanna bracteosa* extract for AGS cells and HuGu cells. After 24 h, the cells were then fixed using ethanol 70% followed by pure ethanol for 15 min and stained with 2 ml of 20 mM Hoechst 33,342 solution for 30 min. There were then washed three times, dried and observed under the fluorescence microscope (Olympus, Japan) at 400× optical amplification.

**Flow cytometry assay**

AGS cells were grown in 35 mm² cell culture plates until reaching to the 80% confluency. On the next day, the cell culture medium was removed and the cells were treated with fresh culture medium containing *Alkanna bracteosa* extract for AGS cell line. AGS cells that not treated with *Alkanna bracteosa* extract were considered as control group. After 24 h, the supernatant of all the treated and non-treated cell groups were discarded, and the cells were washed with PBS (1X). Subsequently, the cells were detached with 1 Zml 0.25% trypsin- 0.02 EDTA (Sigma, USA), at 37°C. Then, DMEM: Ham’s F12 medium supplemented with 10% FBS was added to terminate the trypsinization process, and then gently pipetted to avoid the formation of cell clumps. To continue, the detached cells were transferred into the 15 ml

| Primer name | (5’ → 3’) Sequence |
|-------------|---------------------|
| HER2 forward | 5'-ATCCTCTCATCAAGCGACGGCA-3' |
| HER2 reverse | 5'-CATCGCCTCCGCTAGGGTGTC-3' |
| HSP90AA1 forward | 5'-AAACTGGCGCTCCTGCTTCT-3' |
| HSP90AA1 reverse | 5'-TGGCTGATGGTGTCGTCATCT-3' |
| GAPDH forward | 5'-CTCAATTTCCTGATGTGCAACAG-3' |
| GAPDH reverse | 5'-CTTCCTCTGTGCTTGTGC-3' |
plastic tube and centrifuged at 1000×g for 5 min. Afterward, the supernatant was removed and the cells were resuspended in 500 µl of Annexin V binding buffer. Subsequently, 5 µl of Annexin V-FITC (IQ Products, Netherlands) was added to the cells and incubated for 15 min at 4 °C in the dark place. The cells were then centrifuged at 1000×g for 5 min and the cell pellet were counted-stained with 3 µl propidium iodide (PI) (IQ Products, Netherlands). Finally, the cells were analyzed using BD FACS-Calibur flow cytometry (Becton Dickinson, USA) with emission filters of 530 and 575–610 nm. The extracted data were then analyzed with Cell Quest software (Becton Dickinson, USA) (Hasanzadeh et al. 2017).

DNA fragmentation

The AGS cells were cultured and treated with Alkanna bracteosa extract for 24 h. The cell was then collected in 15 ml plastic tube and centrifuged at 1000×g for 5 min. The DNA was extracted using the SinaPure™DNA kit (Sinaclon, Iran). The nucleosome-sized (200-bp) DNA fragments was separated by gel electrophoresis, on a conventional 2% agarose gel (Orangi et al. 2016).

Statistical analysis

All the received data were reported as an average ± SD and processed in SPSS ver 22. T-test one-way anova was used to determine the significances between difference experimental groups.

Results

Alkanna bracteosa alcoholic extract preparation

After exposure of the areal parts of the plant to the methanolic solution, around 1 gr of alcoholic extract was separated and dried to use for subsequent parts of the researches.

Cytotoxic activity of Alkanna bracteosa alcoholic extract

The results of the MTT assay showed a strong inhibitory effect and dose concentration-dependency of the Alkanna bracteosa extract on the proliferation rate of AGS in comparison to the HuGu cell line. The inhibitory concentrations (IC50) obtained from the AGS cancer cell line and the HuGu normal cell line were 0.8 and 3.2 mg ml−1 during 24 h, 1.6, and 6.4 mg ml−1 during 48 h respectively. Figure 1 demonstrated the comprehensive comparison of cytotoxicity patterns of Alkanna bracteosa extract on the AGS cancer cell line and HuGu normal cell line during 24 and 48 h. This result showed the AGS cells are more fragile than HuGu cells when exposed to the above-mentioned extract of Alkanna bracteosa. Statistical analysis showed significant differences between AGS and HuGu groups treated with the same concentration of Alkanna bracteosa extract.

Quantitative real time PCR study of HER2 and HSP90α genes

The results of qPCR for HER2 and HSP90α genes were shown in Fig. 2. As shown in the Fig. 2a, HER2 gene was significantly down-regulated in AGS cell line treated with 0.8 mg ml−1 dose concentration (P value = 0.0383) compared to control group. By the way, HER2 gene in Fig. 2b showed the up-regulated in 3.2 mg ml−1 dose concentration of HuGu cell line (P value = 0.0021).

Results from qPCR for HSP90α gene demonstrated a significant increase in the expression level in AGS cells treated with 0.8 mg ml−1 dose concentration (Fig. 2c) compared to the control group (P value = 0.0011). Moreover, HSP90α was significantly down-regulated in HuGu cell treatment group treated with Alkanna bracteosa extract at the final concentration of 3.2 mg ml−1 (Fig. 2d) compared to the control group (P value = 0.0069).

Hoechst DNA staining

Induction of apoptosis for AGS and HuGu cell lines was also investigated by microscopic analysis of Hoechst DNA stained cells. Figure 3 showed the untreated control and the Alkanna bracteosa extract treated cells. The 0.8 mg ml−1 dose concentration of Alkanna bracteosa extract for AGS cell line and 3.2 mg ml−1 dose concentration of Alkanna bracteosa extract for HuGu cell line during 24 h demonstrated significant fragmentation within the chromatin structure located in the nucleus of treated cells but it was not found any change in untreated AGS and HuGu cells.

Quantitative investigation of apoptosis by flow cytometry

Flow cytometry was applied to distinguish the apoptotic and necrotic rate of AGS cells. Obtained results from control and treated AGS cells demonstrated that 15.4% of AGS cells treated with Alkanna bracteosa extract at concentration of 0.8 mg ml−1 include 15.4% late apoptosis, about 7.8% early apoptosis, and 13.3% necrosis (Fig. 4a) necrosis compared to control group (Fig. 4b). Furthermore, 1.6 mg ml−1 dose concentration (Fig. 4c) induced 16.8% late apoptosis, 31.8% early apoptosis, and 9.19% necrosis compared to untreated AGS cells (Fig. 4d).
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Apoptosis identification by DNA fragmentation

Formation of the DNA fragmentation in gel electrophoresis by induction of apoptosis in AGS cell line treated with Alkanna bracteosa extract at 0.8 and 1.6 mg ml⁻¹ concentrations was shown in Fig. 5. The apoptosis could be visualized as a fragmentation pattern of 180–200 bp due to DNA cleavage by the activation of a nuclear endonuclease using the 2% standard agarose gel electrophoresis (Loan-nou and Chen 1996; Matalová & Španová 2002).

Discussion

Normal cells can turn into a cancer cells by alteration of the genes that regulate cell growth and differentiation (Croce 2008). By the way, medicinal plants which containing various phytochemicals, such as flavonoids and polyphenol compound, can inhibit tumor cell proliferation and induce apoptosis pathways. Today, most of the plants and their main compounds affect transcription and cell cycle according to different mechanisms (Aiello et al. 2019). For this reason,
the utilization of herbal products with the ability to target the various molecular processes can be an attractive way with the potential of the cancer treatment.

In a study that was reported by Naghibi et al. (2014), the methanolic extract of the native Iranian Alkanna bracteosa could induce cytotoxic effects on MCF-7 cell line. Furthermore, the methanolic extract of Alkanna bracteosa root demonstrated an IC50 dose of 54 µg ml−1 on the MCF-7 cell line (Naghibi et al. 2014). In addition, in a study released by Sevimli-Gur et al. (2010), the cytotoxic effects of naphthoquinones isolated from methanolic extract of Alkanna cappadocica was investigated on twelve human tumorigenic cell lines which including PC-3, HT-29, MDA-MB-231, AU565, Hep G2, MCF7, HeLa, SK-BR-3, DU 145, Saos-2, Hep 3B, LNCaP and two non-tumorigenic cell lines which including NIH3T3 and Vero. The results of that study reported that naphthoquinones components of Alkanna cappadocica could induce cytotoxic effects on the above-mentioned cancer cell lines (Sevimli-Gur et al. 2010).

As it was shown in the present study, the IC50 obtained from the AGS cancer cell line and the HuGu normal cell line were 0.8 and 3.2 mg ml−1 during 24 h and 1.6 and 6.4 mg ml−1 during 48 h respectively. The results revealed that the Alkanna bracteosa extract induced more cytotoxic effects on the AGS cancer cell line than the HuGu normal cell line at the same concentrations, however, the exact mechanism underlying this phenomenon is unclear. Furthermore, we observed higher cytotoxic effect of this extract in 24 compared to 48 h. This might be due to the fact that through the time, the extract is consumed and metabolized by the cells, and the dead cells are substituted by healthier cells.

According to several studies mutations and overexpression of HER2 genes are found in a variety of tumors. It seems that decreased HER2 gene expression level can inhibit tumor growth (Oh & Bang 2019). Furthermore, it was shown that cytotoxic chemotherapy can reduce the expression rate or suppress the HER2 gene (Shu et al. 2018). In a study which was reported by Schroeder et al. (2019), it was shown that HER2 gene expression was inhibited by naphthoquinones (Schroeder et al. 2019). In this study, we found that the HER2 gene was significantly down-regulated in AGS cell line treatment group when exposed to the 0.8 mg ml−1 dose concentration after 24 h of incubation. By the way, elevated HSP90 levels are associated with increased angiogenesis, survival progression, increased metastasis potential, and increased drug resistance (Boroumand et al. 2018). In several cancers, HSP90α was overexpressed and can be secreted outside of tumor cells (Lu et al. 2015). The expression of HSPs is associated with increased resistance to apoptosis and indicates the mechanism of their regrowth (Klimczak et al. 2019). In the present study, a significantly up-regulated expression level of HSP90α in 0.8 mg ml−1 AGS cell line treatment group compared to the control group was shown. We also observed that this gene was significantly down-regulated in 3.2 mg ml−1 HuGu cell line treatment group compared to control groups.

Apoptosis is a complex process responsible for removing the damaged cells from living organisms, and it is in direct correlation with morphological and biochemical changes of the cells. DNA fragmentation occurs during the later stages of the apoptotic process (Majtnerová & Roušar 2018). The endoplasmic reticulum (ER) is an essential organelle for protein synthesis, protein folding, and protein modifications disturbed by physiological and pharmacological stresses and leads to the misfolded proteins (So 2018). However, it is believed that ER distribution associated with oxidative stress and mitochondrial dysfunction, resulting in apoptotic mechanism (Gara et al. 2015). By the way, proteasomes are a group of protein complexes found in all eukaryotes. Their main function is to destroy damaged or unfolded proteins by proteolysis. Shikonin, a component of Alkanna bracteosa extract, plays a direct role in tumor proteasome which can be due to excessive accumulation of incorrectly folded and unfolded proteins in the cells (Gara et al. 2015). Some studies represented that inflammation dispose the development
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of cancer and promotes tumorigenesis process (Greten & Grivennikov 2019).

By the way, several studies reported the anti-inflammatory effects of the *Alkanna bracteosa* extract due to their naphthoquinone structures (Mahmoudi et al. 2012). Naphthoquinones such as alkanin and shikonin and their diversities have many biological properties such as wound healing, anti-inflammatory, anti-tumor, antimicrobial and anticoagulant effects. Furthermore, some shikonin esters have an inhibitory effect on the topoisomerase I enzyme (20, 21). The most probable mechanisms of action of naphthoquinones are including suppression of telomerase activity, DNA damage and ROS production, topoisomerases inhibition, and regulation of p53 receptor as tumor inhibitory factor (Pereyra et al. 2019).

In total, it was found that naphthoquinones such as alkanin and its derivatives can arrest the G1 cell division cycle and induce apoptosis which can be the main reason for its anti-cancer effect on the cells (Huu Tung et al. 2013). Alkanin and shikonin are also considered optical isomers, which arrest the free radical of $O_2^-$. Shikonin have an essential role in wound healing and anti-inflammatory properties, antitumor and antibacterial activity (Sekine et al. 1998; Wang et al. 2019a, b), and inhibit cancer cell growth by interfering in the G0/G1 phase of the cell cycle. They activate the caspase pathway (caspase 3 and caspase 7) which finally leads to apoptosis process (Lan et al. 2014). In a study published by Yang et al. (2009) the role of shikonin in prostate cancer cell death are in direct correlation with ROS production and increased $Ca^{2+}$ levels in the cells (Yang et al. 2009). In a similar study, a type of naphthoquinone could reduce the survival rate of gastric cancer cells through ROS-mediated MAPK and STAT3 signaling pathways (Wang et al. 2019a, b).
In Conclusion, due to the naphthoquinone derivatives of Alkanna bracteosa extract, this extract has been able to significantly reduce the expression of HER2 gene, which is involved in gastric cancer. In addition, Alkanna bracteosa extract can also induce stronger apoptosis effects in treated cancer cells compared to untreated control cells. According to the above-mentioned properties, this plant can be proposed as a valuable candidate for future cancer treatment.

Fig. 4 Flow cytometry results of Alkanna bracteosa treatment in AGS cells during 24 h. AGS 0.8 mg ml⁻¹ treated (a), AGS untreated (b) as control, AGS 1.6 mg ml⁻¹ treated (c), and AGS untreated (d) as control
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Declarations

Ethical statement This article does not contain any studies involving animals performed by any of the authors. This article does not contain any studies involving human participants performed by any of the authors.

Conflict of interest Mina Gholami has no conflict of interest. Zahra Elyasgijri has no conflict of interest. Abdolreza Daneshvar Amoli has no conflict of interest. Parvaneh Farzaneh has no conflict of interest.

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Fig. 5 DNA fragmentation of Alkanna bracteosa treatment after exposure of 0.8 mg ml⁻¹ and 1.6 mg ml⁻¹ respectively
