Advances in Traditional Medicine
https://doi.org/10.1007/s13596-022-00657-z

ORIGINAL ARTICLE

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

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Received: 26 July 2021 / Accepted: 1 July 2022

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 braceteosa* 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,

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Published online: 14 September 2022

Neu), (iii) ErbB3 (HER3), and (iv) ErbB4 (HER4). These receptors play a major role upon activation of downstream pathways which leads to a variety of cellular responses ranging from proliferation, differentiation, and adhesion (Sliwkowski 2001). The ERBB2 gene encodes the HER2 receptor, which is classified as a proto-oncogene. HER2 receptor recognized as a transmembrane glycoprotein containing three main domains which including an extracellular domain, a transmembrane domain, and an intracellular domain (Akiyama et al. 1986; Coussens et al. 1985). The oncogenic defect can be made by a point mutation in the transmembrane domain of the HER2 receptor (Shih et al. 1981). The ErbB signaling network is complex, and despite being widely studied is not fully understood. ErbB2 is one of the key members of the ErbB family. Furthermore, the signaling pathway of ErbB2 comprised of the MAPK and PI3K systems which ultimately leads to transcriptional activation of marked genes (Sidhanth et al. 2018). A high expression level of ErbB2 is reported in 7–34% of patients involved with gastric cancer (Gravalos & Jimeno 2008).

The highly protected stress protein family is known as heat shock proteins (HSPs), which cause by cellular and environmental stress in high temperature conditions, hypoxic damage, and oxidative stress damages (Hoter et al. 2018). HSP90α and HSP90β are two isoforms of human HSP90 (Csermely et al. 2018). HSP90 is responsible for several cellular and physiological functions including cell growth and viability, cell differentiation, cellular stress response, and apoptosis. In fact, this receptor interferes in the functional process of highly expressed proteins that cause malignancy during oncogenesis; and maintains a wide range of mutated or overexpressed proteins in the tumorigenesis signaling pathways (Faridi & Ghahghaei 2018). As molecular chaperones, HSP90 isoforms have wide range of client proteins that are involved in numerous cellular pathways. The interaction list of HSP90 includes kinases such as Akt2, CDKs, PKC, many MAP kinases as well as transcription factors like steroid receptors, BCL-6, CAR, p53, and Oct4 (Hoter et al. 2018).

Apoptosis is a programmed cell death process that is engaged by several signaling pathways such as cellular stress, DNA damage, and the immune system (Carneiro & El-Deiry 2020). In other words, it is a distinct form of cell death in comparison to necrosis; because it is controlled, energy-dependent, and without inflammation (Kerr et al. 1994). It should also be mentioned that the interaction between apoptosis and other signaling pathways can also be effective in cell mortality (Carneiro & El-Deiry 2020). Different classes of regulatory molecules can arrange apoptosis. Some of the biochemical changes include transferring the phosphatidylserine to the outer membrane surface and activation of cysteine aspartyl proteases, called caspases, lead to cell death. However, action mechanisms of apoptosis are typically associated with chromatin density, DNA fragmentation located inside the nucleus, cell compactness, emersion of apoptotic bodies, and reduction of the adhesion tendency to the extracellular matrix (Nikoletopoulou et al. 2013). In order to prevent cancer and other related diseases, the ratio between cell survival and death should be balanced, which apoptosis can be considered as an effective way in this process (Hassan et al. 2014). 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).

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

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% CO_2.

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 10^3 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 HSP90a gene expression

The 10^6 density of the AGS and HuGu cells were then cultured and treated using by *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 mm^2 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 of *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 mm^2 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: Hams 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

Table 1 Primer sequences used in qRT-PCR for HER2 and HSP90 genes

Primer name	(5′→3′) Sequence
HER2 forward	5′-ATCCTCATCAAGCGACGGCA-3′
HER2 reverse	5′-CATCGCTCCGCTAGGTGTCA-3′
HSP90AA1 forward	5′-AAACTGCGCTCCTGTCTTCT-3′
HSP90AA1 reverse	5′-TGCGTGATGTGTCGTCATCT-3′
GAPDH forward	5′-CTCATTTCCTGGTATGACAACGA-3′
GAPDH reverse	5′-CTTCCTCTTGTGCTCTTGCT-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 ^{TM}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 (IC_{50}) 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).

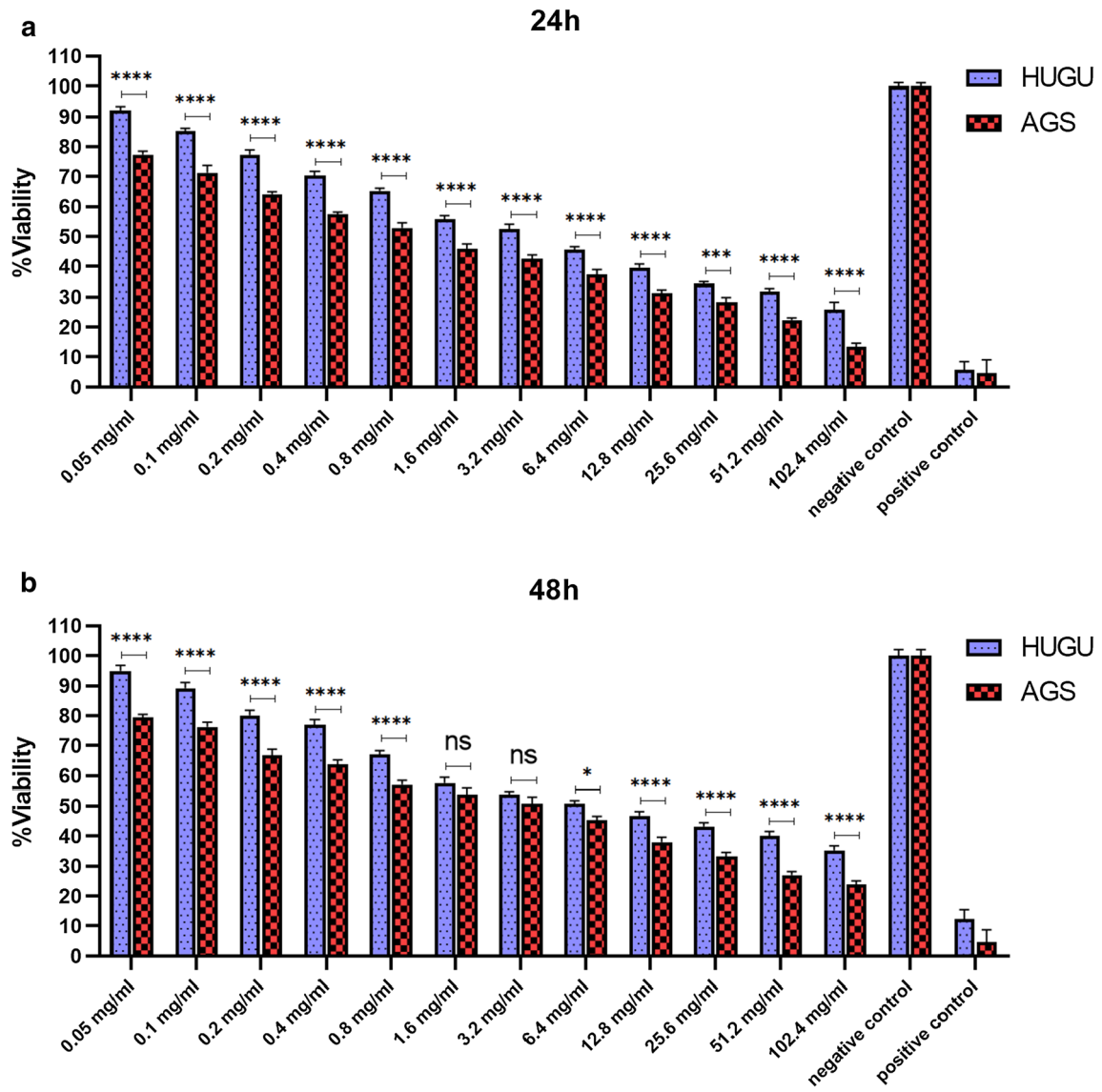

Fig. 1 Comparision between the survival rate of HuGu and AGS cells after 24 h (**a**) and 48 h (**b**). AGS cells represented more death rate than HuGu cells when exposed to different concentrations of Alkanna bracteosa extract. Statistical analysis revealed a significant difference between AGS and HuGu treated groups at the same concentration of Alkanna bracteosa extract and marked by star signs as follows *< 0.05, **< 0.01, ***< 0.001, ****< 0.0001

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 (Loannou 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,

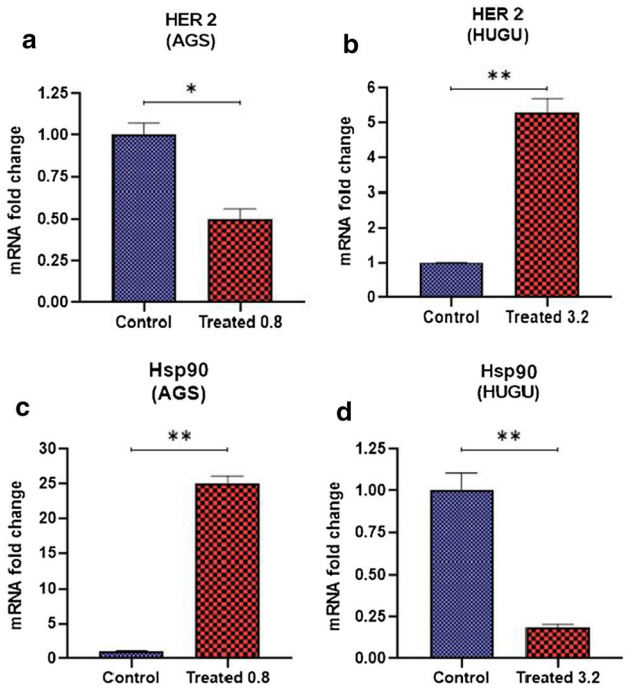

Fig. 2 A representation of HER2 and HSP90α expression levels in AGS (**a**, **c**) and HuGu cells (**b**, **d**) in treated groups (red bars) or untreated control groups (blue bars). HER2 and HSP90α genes were significantly down-regulated (*< 0.0383) (**a**) and up-regulated (**< 0.0011) (**c**) in AGS cells, respectively, while they were up-regulated (**< 0.0021) (**b**) and down-regulated (**< 0.0069) (**d**) in HuGu cells, respectively

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

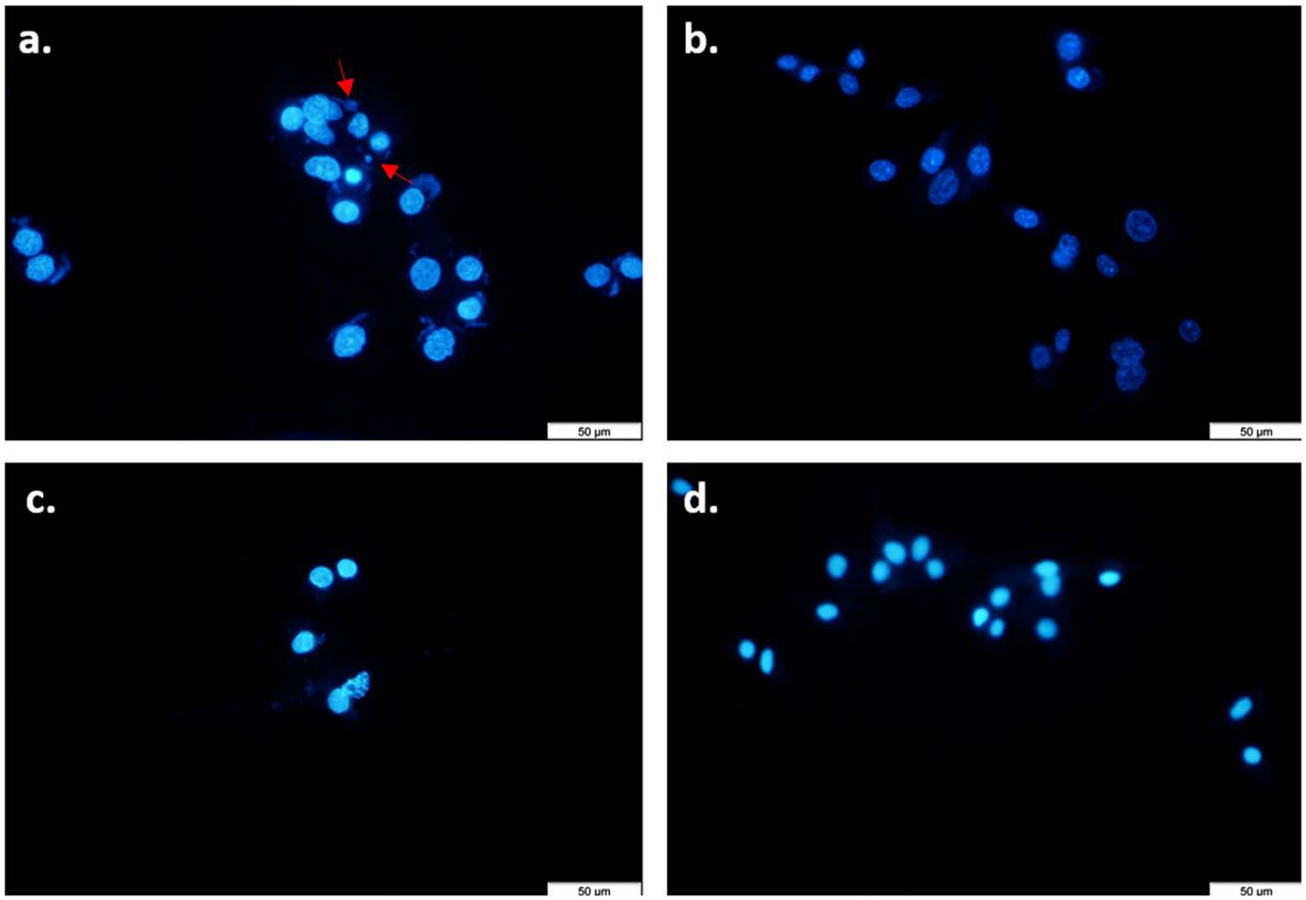

Fig. 3 Hoechst DNA staining. AGS treated cells (**a**), AGS untreated cells (**b**), HuGu treated cells (**c**) and HuGu untreated cells (**d**) when exposed to 0.8 mg ml^{-1} and 3.2 mg ml^{-1} dose concentration of herbal extract for 24 h

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).

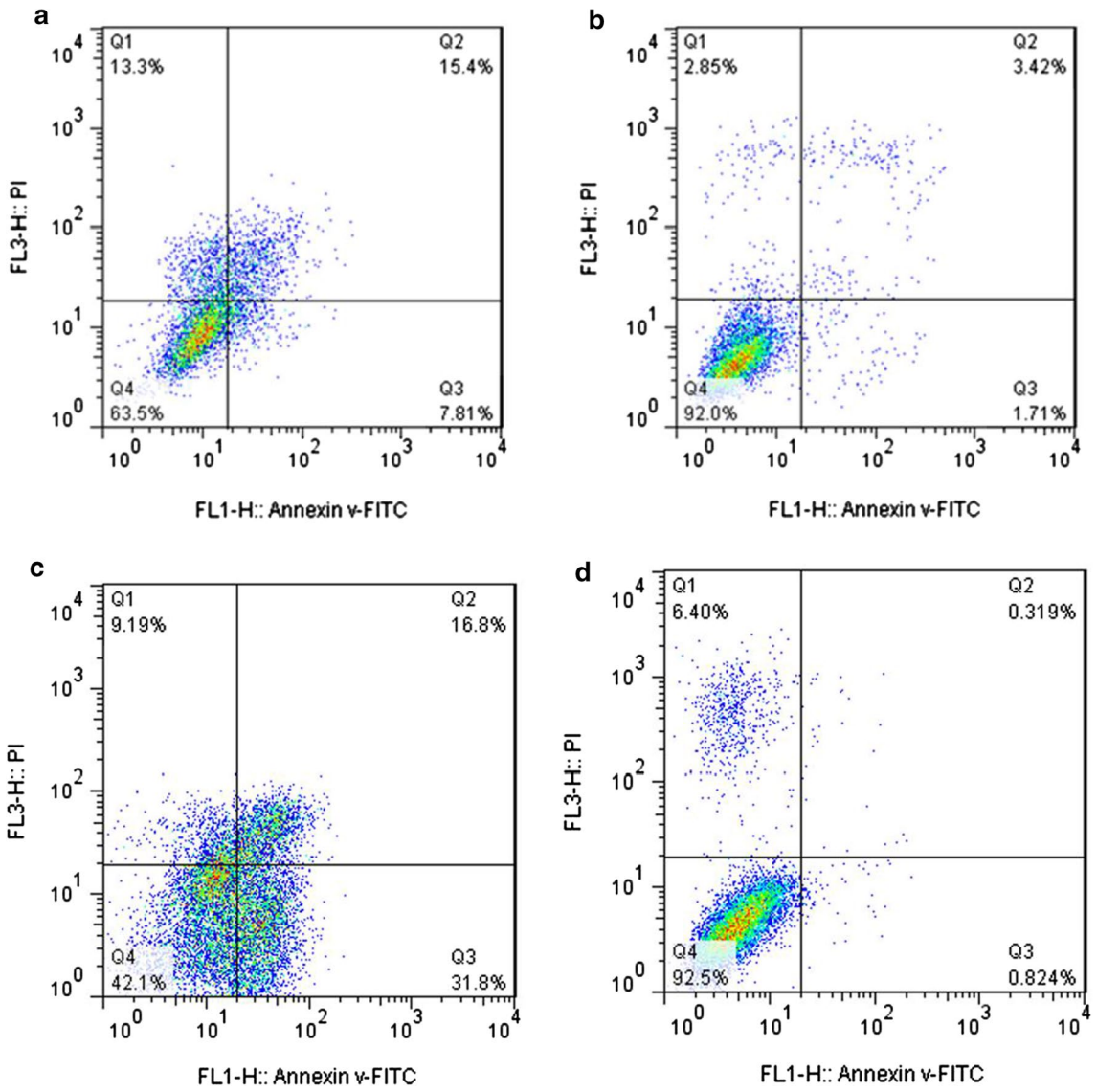

Fig. 4 Flow cytometry results of Alkanna bracteosa treatment in AGS cells during 24 h. AGS 0.8 mg ml^{-1} treated (**a**), AGS untreated (**b**) as control, AGS 1.6 mg ml^{-1} treated (**c**), and AGS untreated (**d**) as control

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.

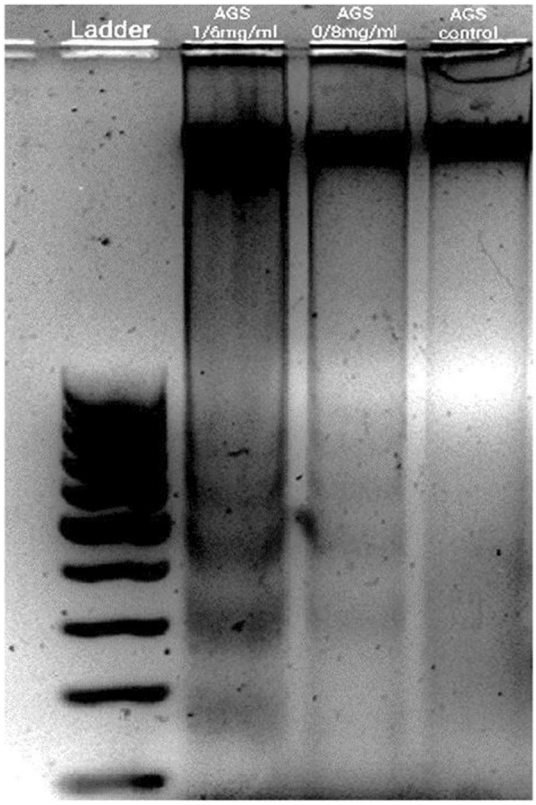

Fig. 5 DNA fragmentation of *Alkanna bracteosa* treatment after exposoure of 0.8 mg ml^{-1} and 1.6 mg ml^{-1} respectively

Acknowledgements The authors appreciate the human and animal cell bank of the Iranian Biological Resource Center (IBRC) for scientific supports of this project. Furthermore, the authors of this study thank the people who had direct collaborations with our team members which including Navid Momenifar and Mohammad Pirouzfar. Dr. Farzaneh, as the first correspondent, lost her life due to the Covid-19 pandemic; but her kind personality, ethic, and scientific supports will stay with us forever.

Funding Funding not available for this article.

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 Elyasigorji has no conflict of interest. Abdolreza Daneshvar Amoli has no conflict of interest. Parvaneh Farzaneh has no conflict of interest.

References

Abuei H, Behzad Behbahani A, Rafiei Dehbidi G, Pirouzfar M, Zare F, Farhadi A (2019) Construction, expression, and purification of p28 as a cell-penetrating peptide with anticancer effects on Burkitt's lymphoma cell Line. Shiraz E-Med J 20(7):e85190

Aiello P, Sharghi M, Mansourkhani SM, Ardekan AP, Jouybari L, Daraei N, Peiro K, Mohamadian S, Rezaei M, Heidari M, Peluso I (2019) Medicinal plants in the prevention and treatment of colon cancer. Oxid Med Cell Longev 2019:2075614

Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T (1986) The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232(4758):1644–1646

Assimopoulou AN, Karapanagiotis I, Vasiliou A, Kokkini S, Papageorgiou VP (2006) Analysis of alkannin derivatives from Alkanna species by high-performance liquid chromatography/photodiode array/mass spectrometry. Biomed Chromatogr 20(12):1359–1374

Boroumand N, Saghi H, Avan A, Bahreyni A, Ryzhikov M, Khazaei M, Hassanian SM (2018) Therapeutic potency of heat-shock protein-90 pharmacological inhibitors in the treatment of gastrointestinal cancer, current status and perspectives. J Pharm Pharmacol 70(2):151–158

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A (2018) Cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68(6):394–424

Carneiro BA, El-Deiry WS (2020) Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol 17(7):395–417

Chowdhury WS (2019) Oncogenes: new targets in cancer treatment. Brac University

Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 230(4730):1132–1139

Croce CM (2008) Oncogenes and cancer. N Engl J Med 358(5):502–511

Csermely P, Schnaider T, So C, Prohászka Z, Nardai G (2018) The 90-kDa molecular chaperone family structure, function, and clinical applications. Pharmacol Therapeut 79:129–168

Faridi N, Ghahghaei A (2018) Hsp90 structure and function in cancer. Gene Cell Tissue 5(3):1–9

Gara RK, Srivastava VK, Duggal S, Bagga JK, Bhatt M, Sanyal S, Mishra DP (2015) Shikonin selectively induces apoptosis in human prostate cancer cells through the endoplasmic reticulum stress and mitochondrial apoptotic pathway. J Biomed Sci 22(1):1–12

Gravalos C, Jimeno A (2008) HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Ann Oncol 19(9):1523–1529

Greten FR, Grivennikov SI (2019) Inflammation and cancer: triggers, mechanisms, and consequences. Immunity 51(1):27–41

Hasanzadeh D, Mahdavi M, Dehghan G, Charoudeh HN (2017) Farnesiferol C induces cell cycle arrest and apoptosis mediated by oxidative stress in MCF-7 cell line. Toxicol Rep 4:420–426

Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N (2014) Apoptosis and molecular targeting therapy in cancer. BioMed Res Int 2014:150845

Hoter A, El-Sabban ME, Naim HY (2018) The HSP90 family: structure, regulation, function, and implications in health and disease. Int J Mol Sci 19(9):2560

Huu Tung N, Du GJ, Wang CZ, Yuan CS, Shoyama Y (2013) Naphthoquinone components from *Alkanna tinctoria* (L.) Tausch show significant antiproliferative effects on human colorectal cancer cells. Phytother Res 27(1):66–70

Kerr JF, Winterford CM, Harmon BV (1994) Apoptosis. Its significance in cancer and cancer therapy. Cancer 73(8):2013–2026

Klimczak M, Biecek P, Zylicz A, Zylicz M (2019) Heat shock proteins create a signature to predict the clinical outcome in breast cancer. Sci Rep 9(1):1–15

Lan W, Wan S, Gu W, Wang H, Zhou S (2014) Mechanisms behind the inhibition of lung adenocarcinoma cell by shikonin. Cell Biochem Biophys 70(2):1459–1467

Loannou YA, Chen FW (1996) Quantitation of DNA fragmentation in apoptosis. Nucleic Acids Res 24(5):992–993

Lu Y, Xu W, Ji J, Feng D, Sourbier C, Yang Y, Qu J, Zeng Z, Wang C, Chang X, Chen Y (2015) Alternative splicing of the cell fate determinant Numb in hepatocellular carcinoma. Hepatology 62(4):1122–1131

Mahmoudi SZ, Seyedabadi M, Esfahani HRM, Amanzadeh Y, Ostad SN (2012) Anti-inflammatory and analgesic activity of Alkanna bracteosa and Alkanna tricophila. Nat Product Res 26(6):564–569

Majtnerová P, Roušar T (2018) An overview of apoptosis assays detecting DNA fragmentation. Mol Biol Rep 45(5):1469–1478

Matalová E, Španová A (2002) Detection of apoptotic DNA ladder in pig leukocytes and its precision using LM-PCR (ligation mediated polymerase chain reaction). Acta Vet Brno 71(2):163–168

Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, Jemal A, Kramer JL, Siegel RL (2019) Cancer treatment and survivorship statistics, 2019. CA Cancer J Clin 69(5):363–385

Mortezaee K, Najafi M, Farhood B, Ahmadi A, Potes Y, Shabeeb D, Musa AE (2019) Modulation of apoptosis by melatonin for improving cancer treatment efficiency: An updated review. Life Sci 228:228–241

Mousavi B, Tafvizi F, Zaker Bostanabad S (2018) Green synthesis of silver nanoparticles using Artemisia turcomanica leaf extract and the study of anti-cancer effect and apoptosis induction on gastric cancer cell line (AGS). Artif Cells Nanomed Biotechnol 46(sup1):499–510

Naghibi F, Khalaj A, Mosaddegh M, Malekmohamadi M, Hamzeloo-Moghadam M (2014) Cytotoxic activity evaluation of some medicinal plants, selected from Iranian traditional medicine Pharmacopoeia to treat cancer and related disorders. J Ethnopharmacol 155(1):230–239

Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N (2013) Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta 1833(12):3448–3459

Oh D-Y, Bang Y-J (2019) HER2-targeted therapies—a role beyond breast cancer. Nat Rev Clin Oncol 17:33–48

Orangi M, Pasdaran A, Shanehbandi D, Kazemi T, Yousefi B, Hosseini B-A, Baradaran B (2016) Cytotoxic and apoptotic activities of methanolic subfractions of Scrophularia oxysepala against human breast cancer cell line. Evid Based Complement Alternat Med 2016:8540640

Papageorgiou VP (1980) Naturally occurring isohexenylnaphthazarin pigments: a new class of drugs. Planta Med 38(03):193–203

Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC (1999) The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. Angew Chem Int Ed 38(3):270–301

Pereyra CE, Dantas RF, Ferreira SB, Gomes LP, Silva-Jr FP (2019) The diverse mechanisms and anticancer potential of naphthoquinones. Cancer Cell Int 19(1):1–20

Pirouzfar M, Amiri F, Dianatpour M, Takhshid MA (2020) CRISPR/Cas9-mediated knockout of MLL5 enhances apoptotic effect of cisplatin in HeLa cells in vitro. EXCLI J 19:170

Salimikia I, Yazdinezhad AR, Golfakhrabadi F, Esfahani HRM (2015) In vitro antioxidant and free radical scavenging activity of four Alkanna species growing in Iran. Pharmacognosy Res 7(1):100

Schroeder R, Sfondouris M, Goyal N, Komati R, Weerathunga A, Gettridge C, Stevens CL, Jones FE, Sridhar J (2019) Identification of new mono/dihydroxynaphthoquinone as lead agents that inhibit the growth of refractive and triple-negative breast cancer cell lines. ACS Omega 4(6):10610–10619

Sekine T, Masumizu T, Maitani Y, Nagai T (1998) Evaluation of superoxide anion radical scavenging activity of shikonin by electron spin resonance. Int J Pharm 174(1–2):133–139

Sevimli-Gur C, Akgun IH, Deliloglu-Gurhan I, Korkmaz KS, Bedir E (2010) Cytotoxic naphthoquinones from Alkanna cappadocica. J Nat Prod 73(5):860–864

Shih C, Padhy L, Murray M, Weinberg RA (1981) Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature 290(5803):261–264

Shu S, Iimori M, Nakanishi R, Jogo T, Saeki H, Oki E, Maehara Y (2018) Changes in HER2 expression and amplification status following preoperative chemotherapy for gastric cancer. In Vivo 32(6):1491–1498

Sidhanth C, Manasa P, Krishnapriya S, Sneha S, Bindhya S, Nagare RP, Garg M, Ganesan TS (2018) A systematic understanding of signaling by ErbB2 in cancer using phosphoproteomics. Biochem Cell Biol 96(3):295–305

Sitarz R, Skierucha M, Mielko J, Offerhaus GJA, Maciejewski R, Polkowski WP (2018) Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res 10:239

Sliwkowski Y (2001) Untangling the ErbB signaling network. 2001. Nat Rev Mol Cell Biol 2:127–137

So J-S (2018) Roles of endoplasmic reticulum stress in immune responses. Mol Cells 41(8):705

Tavakolian S, Goudarzi H, Eslami G, Faghihloo E (2019) Transcriptional regulation of epithelial to mesenchymal transition related genes by lipopolysaccharide in human cervical cancer cell line HeLa. Asian Pac J Cancer Prev 20(8):2455

Teh BT, Fearon ER (2020) Genetic and epigenetic alterations in cancer. Abeloff's clinical oncology. Elsevier, pp 209-224. e202

Wang F, Yao X, Zhang Y, Tang J (2019a) Synthesis, biological function and evaluation of Shikonin in cancer therapy. Fitoterapia 134:329–339

Wang JR, Shen GN, Luo YH, Piao XJ, Zhang Y, Wang H, Li JQ, Xu WT, Zhang Y, Wang SN, Zhang T (2019b) 2-(4-methoxyphenylthio)-5, 8-dimethoxy-1, 4-naphthoquinone induces apoptosis via ROS-mediated MAPK and STAT3 signaling pathway in human gastric cancer cells. J Chemother 31(4):214–226

Yang H, Zhou P, Huang H, Chen D, Ma N, Cui QC, Shen S, Dong W, Zhang X, Lian W, Wang X (2009) Shikonin exerts antitumor activity via proteasome inhibition and cell death induction in vitro and in vivo. Int J Cancer 124(10):2450–2459