Goji berry fruit extracts induce cytotoxicity and apoptotic cell death in breast cancer cells

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ABSTRACT: Anti-cancer agents derived from dietary medicinal plants are of great interest among people. The fruits of the Goji berry (Lycium barbarum L., Fam. Solanaceae), also known as the ‘King of the Berries’, have valuable health benefits and pharmacological properties thanks to their rich content of phytochemicals. Considering the wide range of biological and pharmacological properties of goji berries, it was aimed to investigate the anticancer, antiproliferative and apoptotic cell death activities against human breast adenocarcinoma and carcinoma cells, MCF-7 and T47D, respectively. In the present study, the anticancer activities of the extracts were evaluated using MTT assay, the antiproliferative effects were investigated using bromodeoxyuridine (BrdU) cell proliferation assay, and the apoptotic cell death activities of the extracts were analyzed by immunological-based ELISA. As the main result of this research, it was found that extracts of goji berries significantly decreased the cell viability of breast cancer cells and caused these cells to undergo apoptosis-mediated cell death in a dose- and time-dependent manner. Among the tested extracts, methanol extracts showed the highest anticancer activity against MCF-7 and T47D cells (IC50 = 54.06±0.05 µg/mL and 76.14±0.38 µg/mL, p<0.01, respectively), while the lowest activity was observed in goji berry extracts prepared with dichloromethane (IC50 = 101.05±0.14 µg/mL, p<0.05 and 124.10±0.86 µg/mL, p=0.01, respectively). Moreover, the methanol extracts caused the strongest antiproliferative activity in MCF-7 cells (p<0.05). Regarding the apoptotic cell death potential of the extracts, increased apoptotic cell death was observed in both breast cancer cells, however, apoptotic cell death occurred more strongly in MCF-7 cells than in T47D cells. Consequently, this study suggests that goji berries could be valuable natural sources for the development of herbal formulations against breast cancer. Accordingly, further detailed studies should be conducted to elucidate the molecular signaling pathways and mechanisms of action.

KEYWORDS: Apoptosis; breast cancer; DNA fragmentation; goji berry; proliferation

1. INTRODUCTION

Cancer is one of the most common diseases and disorders that cause a growing health problem worldwide. According to global statistics, cancer affects millions of people globally and is expected to increase rapidly in the following years. Among cancers, breast cancer is one of the three most common cancers around the world, followed by lung cancer and colon cancer. Early detection of breast cancer without detectable distant metastases, along with efficient treatment strategies, is considered curable. Nevertheless, breast cancer is still leading the most common cause of death in both less developed and more developed countries. [1-4]

Dietary medicinal plants such as fruits, vegetables, spices, cereals, and edible tubers/roots contain numerous bioactive compounds that possess valuable health-promoting effects and high nutritional value. These plants contain bioactive secondary metabolites classified as terpenoids (e.g., geraniol, lycopene, indole, piperidine, quinoline, isquinoline), phenolics (e.g., coumarin, lignin, quercetin, genistein, tannins, tannic acids, flavonoids), nitrogen containing compounds (e.g., alkaloids, glucosides, saponins), sulphur-containing compounds (e.g., allinin, phytoalexins, glucosinolates), and others, which provide extremely great opportunities for the management of carcinogenesis. [5,6] Besides carcinogenesis, dietary medicinal plants also play crucial roles in preventing the side effects of reactive oxygen species (ROS), which occur naturally in living organisms. Since preventing the side effects of ROS is one of the most effective strategies to combat diseases and metabolic pathways related to oxidative stress, these plants play significant roles to cope with various diseases including cancer, cardiovascular disease, chronic kidney disease, aging, diabetes, rheumatoid
arthrits, atherosclerosis, and neurodegenerative diseases in the human body. Furthermore, they have great potential for the development of plant-based anti-cancer drugs to develop effective treatment strategies [7-11].

Over the past decades, natural cancer-preventive agents derived from dietary medicinal plants, rather than synthetic agents, have attracted a great deal of interest amongst the people. Previous reports have explored those numerous herbal formulations exert as natural anti-cancer agents with potent antioxidant capacities to reduce free radicals, metal chelators, and singlet oxygen species. Meanwhile, the potential of dietary medicinal plants to serve as natural antioxidants, anticancer, antiproliferative, and apoptotic agents for fighting many types of cancer has been demonstrated in numerous scientific studies [9, 12-15].

Goji berries (Lycium barbarum L., fam. Solanaceae), also defined as ‘King of the Berries’, are distinctly distributed in the Mediterranean area and Southwest and Central Asia, and also widely cultivated in North America, South America, Southern Africa, New Zealand, and Australia. Goji berry has been used in Traditional Chinese Medicine (TCM) for thousands of years to promote overall health. Currently, it has been classified as a nutraceutical food and widely used as a dietary supplement in different beverages and ingredients, especially in China and around the world. Concentrated extracts and infusions have been prepared from goji berry and they have recently been marketed as fruit, drink, ingredient, functional food, and dietary supplements in major supermarkets and direct marketing channels in many countries. [16-18]

Most research on the health benefits of goji berry has demonstrated that goji berry prevents cardiovascular disease, lowers blood sugar and blood lipids, strengthens the immune system, protects against DNA damage, supports brain health by preventing neurodegeneration, and protects the liver and kidney. In addition to the numerous research findings, pharmacological studies also indicate the considerable biological benefits of goji fruit, in particular, anti-aging effect, neuroprotective effect, antioxidant effect, immune-stimulatory effect, cytoprotective effect, hepatoprotective effect, and antidiabetic effects, as it contains a wide range of phytochemicals such as polysaccharides, carotenoids and related compounds (e.g., zeaxanthin, zeaxanthin monopalmitate, lutein, beta-carotene, beta-cryptoxanthin monopalmitate) and other minor constituents. [18-21]

Considering the biological properties of goji fruit, evaluating the cancer prevention potential, determining the antiproliferative capacities, and investigating the apoptotic cell death capacities of the goji berry fruit (L. barbarum L.) against human breast cancer cells are the main objectives of the present research.

2. RESULTS

2.1. Anticancer activity results

The cancer-preventive potential of goji berry (L. barbarum) fruits was assessed towards MCF-7 and T47D human cancer cells, and HUVEC control cells. The extracts of goji berry exerted remarkable cytotoxic and anticancer potential against the tested cancer cells in a dose and time-dependent manner. The anticancer activity results were presented in Table 1, as IC_{50} (µg/mL) values at a concentration of 100 µg/mL after 72 hours.

As can be seen in Table 1, all of the goji berry fruit extracts tested caused much greater cytotoxicity to MCF-7 than to T47D cells. The methanol extracts of L. barbarum were found higher anticancer activity than those of the water and dichloromethane extracts against all the cancer and control cells. The methanol extracts exhibited the strongest anticancer activity towards MCF-7 cells (IC_{50} = 54.06±0.05µg/mL, p< 0.01), while dichloromethane extracts exhibited the weakest anticancer activity against both MCF-7 and T47D human cancer cells (IC_{50} = 101.05±0.14µg/mL, p< 0.05 and 124.10±0.86 µg/mL, p< 0.01, respectively).

2.2. Antiproliferative activity results

Antiproliferative activities of various extracts from the fruits of goji berry were tested on human breast cancer cells. The effects of the extracts on cell viability in MCF-7 and T47D cancer cells were analyzed as dose-and-time dependently. Cells were counted at different time intervals, 24, 48 and 72 hours, and different concentrations of the treated extracts. The percent cell (%) of the cancer cells was compared with the control cell (HUVEC), and shown in Figure 1, after being treated with the extracts of goji berry for 48 hours. The results represent the mean ± SEM of three independent experiments performed in triplicate.
Table 1. Anticancer activities of goji berry extracts against human breast cancer cells

<table>
<thead>
<tr>
<th>Human cancer cells</th>
<th>Extracts</th>
<th>IC_{50} values* (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>MeOH</td>
<td>54.06±0.05**</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>68.30±0.19**</td>
</tr>
<tr>
<td></td>
<td>CH₂Cl₂</td>
<td>101.05±0.14*</td>
</tr>
<tr>
<td>T47D</td>
<td>MeOH</td>
<td>76.14±0.38**</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>72.18±1.10*</td>
</tr>
<tr>
<td></td>
<td>CH₂Cl₂</td>
<td>124.10±0.86**</td>
</tr>
<tr>
<td>HUVEC</td>
<td>MeOH</td>
<td>47.02±1.28**</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>50.18±0.76**</td>
</tr>
<tr>
<td></td>
<td>CH₂Cl₂</td>
<td>69.84±0.91*</td>
</tr>
</tbody>
</table>

Doxorubicinb< 6.50±0.08

a Values were expressed as IC_{50} ± SD from three independent experiments (n=3).
b Doxorubicin, positive control.
*p value of < 0.05; **p value of < 0.01.

Figure 1. Cell viability percentage of MCF-7 and T47D cancer cells
HUVEC cells were used as control and set as 100%.
[(a): methanol extracts of goji berry fruits; (b): distilled-water extracts of goji berry fruits; (c): dichloromethane extracts of goji berry fruits]

As shown in Figure 2, the extracts were able to decrease cell proliferation in both breast cancer cells, even at the lowest concentration (10 µg/mL) and shortest exposure time. Similar to the MTT analysis, the extracts caused more cell death in MCF-7 than in T47D cells. The extract of goji berry prepared with methanol caused the highest cell death in MCF-7 cells with 20% cell viability, while the water extract caused the highest cell death in T47D cells with 32% cell viability, closely followed by the water extract. In other words, dichloromethane extracts were observed to be the weakest antiproliferative extracts against the MCF-7 and T47D breast cancer cells, with 45% and 57% cell survival, respectively (Figure 1).
2.3. Apoptotic activity results

The level of apoptosis was measured by ELISA assay based on the antigen-antibody complex in MCF-7 and T47D breast cancer cells treated with the extracts at different concentrations. In the present study, doxorubicin was used as a positive control, whilst the cells that were not treated with the goji berry extracts were used as a negative control. It was found that the extracts obtained from the fruits of goji berry increased apoptotic cell death in both breast cancer cells, depending on the concentration of the treated extract. The changes in apoptotic cell death based on apoptotic DNA fragmentation are shown in Table 2 as relative absorbance values for each cell line.

Table 2. Effects of the goji berry extracts on apoptotic cell death in breast cancer cells

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Absorbance ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeOH</td>
</tr>
<tr>
<td>MCF-7 Cells</td>
<td></td>
</tr>
<tr>
<td>0 μg/ml b</td>
<td>0.946±0.014</td>
</tr>
<tr>
<td>10μg/ml</td>
<td>2.601±0.026</td>
</tr>
<tr>
<td>50μg/ml</td>
<td>3.804±0.068</td>
</tr>
<tr>
<td>100μg/ml</td>
<td>4.517±0.019</td>
</tr>
<tr>
<td>200μg/ml</td>
<td>5.620±0.005</td>
</tr>
<tr>
<td>Doxorubicin c</td>
<td>5.186±0.044 (at 200μg/mL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Absorbance ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeOH</td>
</tr>
<tr>
<td>T47D Cells</td>
<td></td>
</tr>
<tr>
<td>0 μg/ml b</td>
<td>0.892±0.006</td>
</tr>
<tr>
<td>10μg/ml</td>
<td>1.975±0.017</td>
</tr>
<tr>
<td>50μg/ml</td>
<td>3.560±0.094</td>
</tr>
<tr>
<td>100μg/ml</td>
<td>4.276±0.046</td>
</tr>
<tr>
<td>200μg/ml</td>
<td>5.049±0.078</td>
</tr>
<tr>
<td>Doxorubicin c</td>
<td>4.975±0.062 (at 200μg/mL)</td>
</tr>
</tbody>
</table>

* Data are presented as mono- and oligonucleosome enrichment ± standard deviation, expressing DNA fragmentation in cells after 48 hours of treatment
* Cells treated with 0 μg/ml of the extract was used as a negative control.
* Doxorubicin (100 μg/ml) was used as a positive control.

Consistent with the anticancer and antiproliferative activity results, apoptotic cell death was found to be more severe in MCF-7 cells compared with T47D cells. In addition, it was found that the tested methanol extracts at a concentration of 200 μg/mL were able to trigger apoptosis by increasing mono and oligonucleosome formation more effectively than doxorubicin at the same dose in MCF-7 and T47D cells. The results of the studies also include that the same dose did not affect cell viability in HUVEC control cells. The amount of mono and oligonucleosomes observed based on apoptotic DNA fragmentation in the cells was highest in methanol extracts, whereas it was the lowest in dichloroform extracts.

3. DISCUSSION

Cancer has become one of the most important health problems of today, both in developed and developing countries. Cancer is a multifactorial disease in terms of its mechanisms of origin, development, and outcomes. It is characterized by different processes in affected individuals and exhibits great heterogeneity and diversity at the cellular and molecular levels. In recent years, many cancer patients are turning to alternative treatments due to the development of drug resistance, non-tumor specific treatment, the cost of cancer treatment, and similar reasons [22-23]. In this context, medicinal and aromatic plants with their rich bioactive compounds and secondary metabolites are of undeniable importance as “complementary medicine (phytotherapy)” in the treatment and prevention of many diseases, especially cancer. [10-12, 15]

Previous studies conducted with the goji berry found potent antiproliferative properties of the extracts against breast cancer cells, with the IC₅₀ value of 0.75 mg/mL for 96 hours treatments, as compliant with the results obtained from the present research. Besides, the tested goji berry extract was found to reduce cell proliferation in the breast cancer cells to 70%, 55.7% and 51.4% after 24, 48 and 96 h, respectively at the 1 mg/mL concentration. [24] Furthermore, previous reports suggest that polysaccharides isolated from goji fruit are able to inhibit proliferation by de-regulating cell cycle proteins such as p53, p21, p27, cyclins and cyclin-dependent kinases. [24-26] In another research performed by Luo et al. (2009), the apoptosis-inducing effect of goji berry polysaccharides was shown against prostate cancer cells, including PC-3 and DU-145. The
inhibition ratios of the extracts were determined 91.0\% and 92.5\% on PC-3 and DU-145, respectively, at the concentration of 1 mg/mL, which is more similar to the current research. As consistent with the inhibition results of the extracts on cell growth, and significant alterations in the expression of pro- and anti-apoptotic proteins were also demonstrated as 40\% for PC-3, and 21.3\% for DU-145 (p< 0.01). [27] Similarly, a colorectal cancer cell line, CT26-WT, was treated with different concentrations of extracts of goji berry, and remarkably increased cytotoxicity of dendritic cell-mediated cytotoxic T-lymphocytes against the CT26-WT cells was observed in this research. Accordingly, goji berry can provide significant benefits to fight cancer cells, apart from its ability to use immunoregulation to target tumor cells. [28] Consistent with the effect observed in colorectal cancer cell lines, goji berry seems to cause cytotoxicity and promote apoptosis of human cervical cancer (HeLa) by both preventing cell cycle progression with the inhibition of 35\% (p< 0.05) at the dose of 6.25 mg/ml, and arresting cell cycle in S phase (33.5–59.4\%). [29] Based on the findings obtained from the previous works, polysaccharides and carotenoids are the main groups of bioactive phytochemicals found in goji berries. Indeed, these components are known to have promising therapeutic effects in many diseases, and are therefore likely responsible for growth inhibition in cancer cells, as shown by previous studies. [18-20, 24-29]

It is well-known that cancer cells have different characteristics properties from healthy cells. The death patterns, metabolic processes and signal pathways in cancer cells also differ from non-cancerous cells. As one of the characteristics hallmarks of cancer, angiogenesis is the formation of new blood vessels from pre-existing blood vessels. It is a dynamic process involving cell proliferation, migration, adhesion, tumor growth, and tube formation in endothelial cells. Even though, this process tightly balanced by many factors, molecules and signaling pathways, it is mainly regulated by the production of angiogenic stimulators including vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF-\(\alpha\)), which acts as pro-angiogenic factors. A shift in this balance causes to pathological uncontrolled angiogenesis in cancer cases. Human Umbilical Vein Endothelial Cells (HUVECs) are isolated from human umbilical cord, and used as a model system for angiogenesis. [30-32] Therefore, the HUVEC cell line was used to compare with cancerous cell lines (MCF-7 and T47D cells), when examining anticancer activities of the goji berry extracts in this research. However, a few studies are focusing on the anti-cancer activities of the goji berry, no study has yet been performed to investigate anti-breast cancer activity in combination with the proliferative and apoptotic activities of different extracts of \(L. \ barbarum\). Therefore, this study is the first investigation aimed to demonstrate the anticancer, antiproliferative, and apoptotic activities of goji berry, which are also important in the field of complementary medicine. Thanks to the results of this study, a chemopreventive agent has been added to the literature, which could be important for breast cancer research and the pharmaceutical field.

4. CONCLUSION

Overall, goji fruit possesses a variety of pharmacological properties that have been demonstrated in various \textit{in vivo} clinical studies in animals and human. In the current research, the anticancer, antiproliferative, and apoptotic cell death properties of different extracts from goji berry (\(L. \ barbarum\ L\.) were analyzed by \textit{in vitro} assay systems. The results suggest that goji berry may be a good candidate for the development of plant-based formulations for cancer prevention and inhibition of cell proliferation of breast cancer cells. Accordingly, it can be concluded that \(L. \ barbarum\) is a valuable natural source for therapeutic purposes. Further in vivo studies and clinical trials are needed to clarify the exact mechanisms of these effects.

5. MATERIALS AND METHODS

5.1. Collection of plant material

The fruits of goji berry (\textit{Lycium barbarum}) used herein were collected from a 3-year-old experimental plantation located in the region of southwestern Turkey. The fruits were dried in the shade under laboratory conditions. The herbarium record of the plant samples was kept in the Department of Biology, Gaziantep University, Turkey (GAUNHERB 1218A). The plant material and fruits of the goji berry are shown in Figure 2.
5.2. Preparing the extracts

Dried whole fruits of goji berry were extracted using the maceration method described previously. [33-34] For extraction, the fruits were frozen with liquid nitrogen and then powdered. 10 g of the powdered samples were extracted with methanol (MeOH), distilled water (dH₂O), and dichloromethane (CH₂Cl₂). The solution was then filtered and methanol was evaporated in a rotary system. The obtained extracts were kept in a freezer at -20°C until further analysis. The extracts were prepared by dissolving in dimethyl sulfoxide (DMSO) at the highest concentration of 1 mg/mL.

5.3. Human cancer cell lines and culture conditions

MCF-7 (breast adenocarcinoma) and T47D (breast carcinoma) human cancer cells, and non-tumorous HUVECs (human umbilical vein endothelial cells), obtained from the American Type Culture Collection (ATCC, USA) were used to determine the potential anticancer and cytotoxic activities of goji berry extracts. The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM): Ham’s F12 nutrient medium (1:1) (ThermoFisher Scientific) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin in the flasks at 37°C in a humidified CO₂ (5%) incubator. Cell culture conditions and supplements were used as described in previous publications. [33-35]

5.4. Anticancer activity

For evaluation of the anticancer activity of the goji berry fruit extracts, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described by Mosmann (1983) with slight modifications [36,37]. Accordingly, the cells were seeded in a 96-well microplate at a density of 5x10^3 cells/well in 200 µl DMEM: Ham’s F12 growth medium and then incubated at 37°C. The cells were exposed to various concentrations ranging from 0.01 to 1 mg/ml of the extracts for 24, 48, and 72 hours. Subsequently, the metabolic activity of the living cells was measured at a wavelength of 570 nm using a Thermo Lab System 408 Multiskan multiplate spectrophotometer. Doxorubicin was used as a positive control, whilst DMSO was used as a negative control. The dose response curve was plotted and IC₅₀ values (µg/mL) were calculated for each cell line.

5.5. Antiproliferative activity

Bromodeoxyuridine (BrdU) cell proliferation assay was used to measure the antiproliferative activities of the goji berry fruit extracts. In this assay, DNA synthesis in proliferating cells was assessed by measuring BrdU incorporation using a commercial cell proliferation ELISA system (Roche Molecular Biochemicals, Manneheim, Germany) with some modifications[37,38]. Briefly, the cells were plated at a density of 5x10^3
cells/well on a 96-well microplate and incubated at 37°C for 24 hours. The cells were then treated with different concentrations (0.01-1 mg/ml) of the extracts, and incubated for 48 hours. After incubation, the cells were exposed to BrdU, according to the manufacturer’s protocol. The absorbance was measured at 450 nm wavelength using a Thermo Lab system 408 Multiskan multiplate spectrophotometer.

5.6. Apoptotic activity

Immunological-based ELISA (enzyme-linked immunosorbent assay) assay was performed to detect apoptotic cell death in cancer cells treated with fruit extracts of goji berry at various concentrations. [39] In brief, culture media containing 5x10^4 cells/mL were incubated with different concentrations (0.01-1 mg/ml) of the extracts for approximately 24 hours after exposure. Nucleosomes in the cytoplasm of apoptotic cells were determined using the CDD-ELISA kit (Cell Death Detection ELISAPLUS, Roche). The absorbance values at 405 nm wavelength were averaged, and the relative absorbance values were calculated according to the equation given below. The relative absorbance value obtained from the equation expresses the ratio of the amount of mono- and oligonucleosomes in each sample to that in the control.

Relative Absorbance Value = \( \frac{A_1}{A_2} \)

\( A_1 \) represents the average of the absorbance values of the cells treated with the extract and \( A_2 \) represents the average of the absorbance values of the control cells.

5.7. Statistical analyses

The results were presented as the mean and standard deviation of the mean (mean±SD), and statistical analyses were conducted using GraphPad Instant version 3.05 (GraphPad Software Inc., CA, USA). The measurements and calculations were evaluated using the one-way ANOVA and Tukey’s Multiple Comparison post-test. A *p-value of <0.05 was considered statistically significant and **p <0.01 was considered highly significant.

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Author contributions: Concept, Design, Materials, Data Collection and/or Processing, Analysis and/or Interpretation, Literature Search and Writing – S.G.

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