Original Article

Anticancer Studies of Iraqi-cultivated *Cydonia Oblonga* From the Family of Rosaceae

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ABSTRACT

Background: *Cydonia oblonga* Miller is a plant that provides numerous phytochemical benefits that could be used in medicine, such as flavones, flavonols, and their glycosides, in addition to different vitamins and minerals.

Objectives: This study evaluates the in vitro cytotoxic activity of *C. oblonga* ethyl acetate of two plant parts (fruit and seeds) against human colon cancer cells (Caco-2) using the MTT assay method.

Methods: In this study, two parts of the *C. oblonga* plant were used (seeds and fruit) and extracted by using different organic solvents, such as 98% hexane, 80% ethanol, and ethyl acetate. The ethyl acetate extract of both parts was used to evaluate the cytotoxic activity of *C. oblonga* on human colon cancer cells (Caco-2) by using the MTT assay method.

Results: The ethyl acetate extract of seeds had no considerable cytotoxic effect ($P>0.1$), while the fruit extract showed a significantly high antiproliferative effect compared to the oxaliplatin anticancer drug ($P<0.1$).

Conclusion: The fruit ethyl acetate extract of the quince plant gives significant anticancer activity against colon cancer cells as compared to seed extract which showed no effect.

Keywords: Anticancer, Caco-2, Cydonia, Ethyl acetate, MTT

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Introduction

Nature has given a tremendous amount of herbal therapeutic items over the last thousand years, and modern medicinal drugs isolated from natural sources are astounding in their effectiveness. Many of these isolations have been based on the use of drugs that are commonly used in conventional medicine. Traditional medicinal systems are still used in healthcare, with about 80% of the world's population relying on traditional primary medical items as their major source of treatment (Owolabi et al., 2007). *Cydonia oblonga* Miller is a flowering plant innate to the Mediterranean region and Minor Asia. It is a member of the Rosaceae family, Maloideae subfamily, Pireas tribe, and the genus Cydonia. Meanwhile, it is a constitutional plant. In the United States and many other countries worldwide, cancer is a significant public health issue. Nowadays, colorectal cancer is the third most prevalent cancer in the United States, diagnosed in men and women. Studies on the benefits of organic compounds produced from plants in the prevention and treatment of cancer have grown significantly in recent years. Modern research suggests that polyphenols may be crucial dietary components for cancer chemoprevention. Silva and co-workers' studies over the last few years have shown that *C. oblonga* Miller, often known as quince, is an excellent, risk-free, and reasonably priced natural source of many types of phenolic compounds, including caffeoylquinic acids (Silva et al., 2002). These substances may give a chemical basis for health benefits, including the high antioxidant potential by scavenging the free radicals (Babaahmadi Milani et al., 2020). In folk medicine, quince leaves and fruit have health benefits for cardiovascular disease, hemorrhoids, bronchial asthma, and cough (Marcia et al., 2010). Accordingly, this study evaluates the in vitro cytotoxic activity of *C. oblonga* ethyl acetate of two plant parts (fruit and seeds) against human colon cancer cells (Caco-2) by using the MTT assay method.

Materials and Methods

Fresh unmatured fruit of *C. oblonga* was cultivated from Iraqi farms in the middle of September. Seeds and fruit (pulp and peel) were separated and left to dry at room temperature between 4-7 days. Subsequently, the dried parts were ground and weighed.

Plant extraction

An amount of 100 g of dried powder plant parts (seeds and fruit) was extracted by maceration with 2.5 L of n-Hexane (99.8%, Sigma-Aldrich) for 5 days to defat the plant. The defatted parts were then macerated with ethanol (80%, Merck) for another 5 days. Both extracts were concentrated to about 50 mL using a rotary evaporator at 40 ˚C. After that, the ethanol fraction was partitioned with ethyl acetate (Fisher Scientific), and this final extract was used to determine the cytotoxic activity.

Identification of phenolic compounds

The phenolic compounds found in ethyl acetate extracts of both seeds and fruit were identified by analytical thin layer chromatography and reverse-phase high-performance liquid chromatography system C18 column (250×4.6 mm inner diameter; Shimadzu) with a degasser (DGU-20A), by using different solvent systems for seeds and fruit (pulp and peel) (Han & Row, 2011).

Cell culture preparation

To investigate the cytotoxic activity of the ethyl acetate extracts of both seeds and fruit of Iraqi cultivated *C. oblonga*, human colon cancer cells (Caco-2 cell line) were used. The cells were obtained from the cell bank of the Tissue Culture Research Center at Al-Mustansiriyah University, College of Pharmacy.

At first, Caco-2 cells were maintained in 1% penicillin-streptomycin (Fisher Ltd. USA), 10% fetal bovine serum (Fisher Ltd. USA), 1% L-glutamine along with Dulbecco's modified eagle medium (DMEM) (Capricorn scientific, Germany) to prevent contamination. Then a 750-cm^2 flask was used for the growth of cells and preserved in an incubator with 5% CO_2, and 95% humidified air at 37 ˚C. In the next step, Caco-2 cells were passaged under sterilized conditions after they reached 90% confluence. Then 5 mL of phosphate buffered saline solution was used to wash grown cells and incubated for 2 min at 37 ˚C in trypsin solution (Fisher Scientific, USA) for detachment of cells from the bottom of the flask (trypsinization). In addition, an equivalent volume of development media was added to the cell suspension before being transferred to a 50-mL conical tube. Then at 1200 rounds per min, cells were centrifuged for about 5 min. The supernatant was discarded and the pelleted cells were re-suspended in fresh enriched growth media. The last step was the counting of cells under a microscope using a hemocytometer (Shi et al., 2012; Rodgers & Grant, 1998).

After trypsinizing, a 75-cm^2 confluent flask, the cell suspension was centrifuged for approximately 5 min at 1200 rounds per min. The cell pellet was then resuspended in 4 mL of freezing medium and aliquots (1 mL) were added to cryovials. For 24 h, the cells were kept at -80 ˚C. The cells were then quickly frozen at 37 ˚C and 10 mL of newly prepared growth medium was added. The cells were then collected during centrifugation and resuspended in 25 mL of fresh development medium before being transferred to a 75-cm^2 flask (Shi et al., 2012).

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium) assay was used to determine the effect of Iraqi *C. oblonga* ethyl acetate extracts (seeds and fruit) on colon cancer cell viability. A total of 100 µL of cell suspension was dispensed into 96-well flat-bottom tissue culture dishes at a concentration of 5×10^3 cells per well, and cultured for 24 h in usual conditions; 48 h at 4×10^3 cells per well, and 72 h at 3×10^3 cells per well. The cells were treated with (1.5, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL) doses of both extracts after 24 h. After a 24-h, 48-h, and 72-h recovery period, the medium was removed from cells and 30 µL of MTT solution (composed from 3 mg/mL in phosphate-buffered saline) was added to each well and incubated for 4 h at 37 ˚C. The well plate was then carefully held, tapped on paper, and the medium gently removed. The following step was the addition of 100 µL of growth media as a control and each well received 100 µL of dimethyl sulfoxide solution (Fisher Ltd. USA). They were then kept in dark conditions at room temperature for about 15-20 min. The determination of absorbance to each well was done using multi-scan reader equipment operating at a wavelength of 540 nm, and absorbance was modified using a 650 nm wavelength (Vijayarathna & Sasidharan, 2012). The test was repeated three times and the percentage of inhibition rate was calculated as follows (Equation 1):

1. IR%=A−B/A×100

where “IR” is the percent inhibition of development, “A” denotes the control absorbance, and “B” denotes the test absorbance.

Following in vitro MTT studies, the half maximal inhibitory concentration (IC_{50}) value denotes the concentration of tested ethyl acetate (seeds and fruit) extract that inhibits 50% of cell viability. The IC_{50} value was determined following the data obtained from the in vitro MTT assay after 27-h (Sebaugh, 2011).

Data analysis

The one-way analysis of variance with Tukey (GraphPad Prism software, version 22) was used to compare groups within the same plate and non-linear curve fitting software was used to determine the statistics of both (seeds and fruit) extracts for MTT assay and IC_{50} values on Caco-2 cells. $P<0.05$ were considered statistically significant (Ala et al., 2018).

Results

Phenolic compounds

Apigenin, luteolin, and kaempferol were identified in seeds of ethyl acetate extract. While astragalin glycoside and isorhamnetin were found in fruit extract with the last one identified for the first time in *C. oblonga* according to previous studies. Apigenin, luteolin, astragalin, and isorhamnetin were isolated, the yield was 1.5%, 1.25%, 1.6%, and 1.3% respectively. After that, those isolated compounds were structurally elucidated by ultraviolet, Fourier-transform infrared spectroscopy, proton nuclear magnetic resonance, and carbon-13 nuclear magnetic resonance.

Cell viability assay

The percentage of cell death of Caco-2 cell lines by seeds and fruit (pulp and peel) extracts is as follows. Different extracts concentrations (100, 50, 25, 12.5, 6.25, 3.125, and 1.5 µg/mL) were used compared to the anticancer drug, oxaliplatin, to evaluate the cytotoxic activity of Iraqi cultivated *C. oblonga* on human Caco-2 cell lines by MTT assay method over 24, 48, and 72 hours.

According to Figure 1, fruit extract highly improved the percentage of cell death of Caco-2 cell lines and this effect increased over time. However, no effect was seen when seed extract was used. In addition, the percentage of cytotoxic activity was observed at high concentrations (100 µg/mL) which was 42.47% at 24 h and increased over time up to 66.89% at the end of 72 h compared with that of the reference standard, oxaliplatin (Jefferson Impex Private Limited, India), which started from approximately 43%, 72% and 92.22% at 24, 48, and 72 h, respectively, at the same concentration at $P<0.0001$.

Meanwhile, according to Figure 2, the effect of the concentration on cell growth inhibition percentage clearly shows that as the concentration of fruit extract increased, there was more inhibition. Therefore, the maximum cell death was at 100 µg/mL which was at 66.89% compared

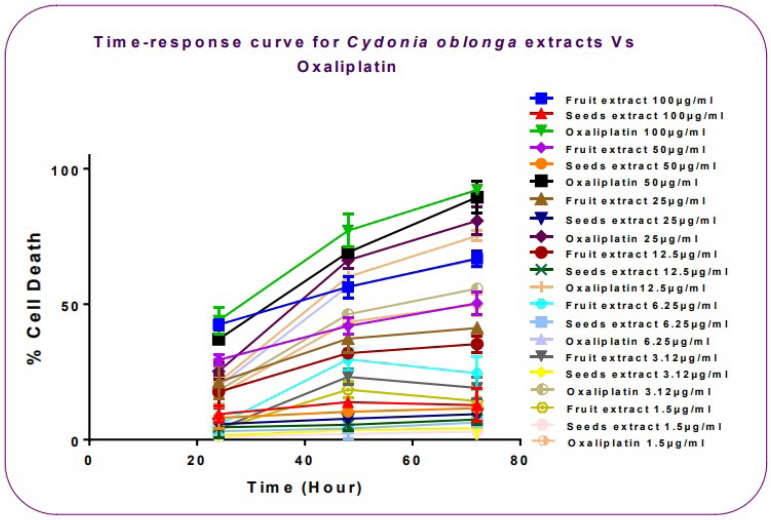

Figure 1. In vitro assessment of cell death percentage of Caco-2 cell line

Notes: Cells were evaluated by MTT assay by using a 96-well plate after 24, 48, and 72 h treated with 1.5, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL fruit and seeds extract. Data is shown as a percentage of Mean±SE of the mean of cell death of 3 separate experiments. Treatment is significantly different from the two fractions P<0.0001.

to the standard anticancer drug at 92.22%. All the results were recorded at the end of 72 h. Conversely, seed extract had no inhibition effect starting from 2.98% at 1.5 µg/mL and ending with a very slight elevation to 12.7% at 100 µg/mL at P<0.05.

Half maximal inhibitory concentration value

IC_{50} was identified by using non-linear regression analysis for fruit and seed extract of Iraqi cultivated *C. oblonga*. From the data obtained in the dose-response curve in Figure 3, IC_{50} for the fruit extract was 68.67 µg/mL and for the seeds extract it was more than 100 µg/mL, compared to the IC_{50} of the control, oxaliplatin, which was at 7.95 µg/mL.

Discussion

In America and other countries, cancer is considered the main health problem facing the community. Choosing the specific therapy to treat many kinds of cancer is

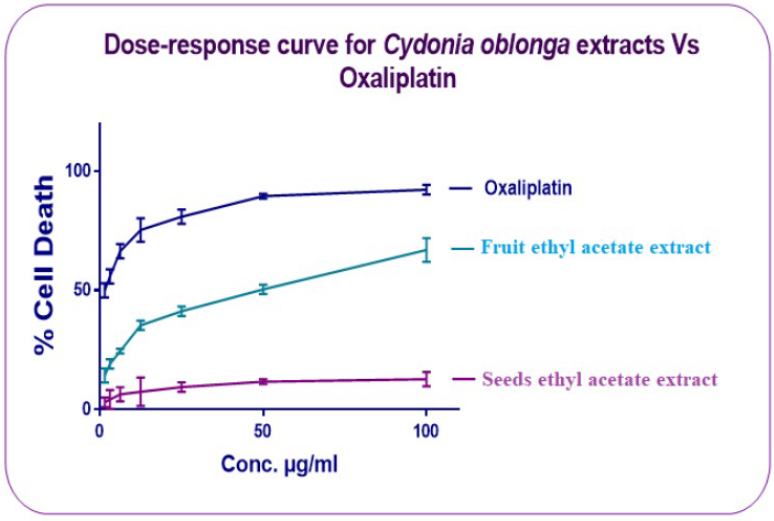

Figure 2. In vitro comparison of cell death percentage of Caco-2 cell lines at 72 h

Notes: The cells were treated with different concentrations of fruit and seed extracts and oxaliplatin (control). The measurement of absorbance was at 540 nm (reference wavelength 650 nm) using a microplate reader. Fruit extract has a strong inhibition ability on Caco-2 cell lines (P<0.05) vs seeds extract.

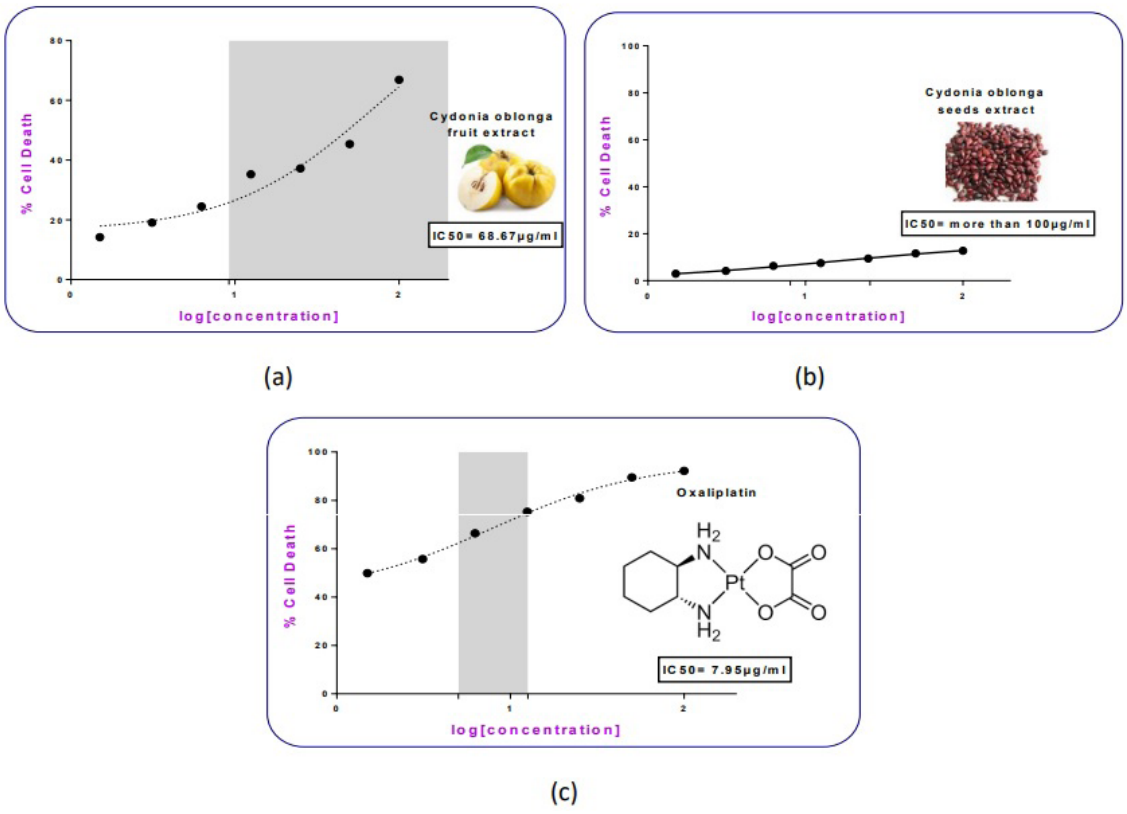

Figure 3. Dose-response curves of half maximal inhibitory concentration for Caco-2 cell lines

Notes: The cells were treated with fruit extract (a) and seeds extract (b); meanwhile, oxaliplatin was considered control (c), for 72 h with 100, 50, 25, 12.5, 6.25, 3.12, 1.5 µg/mL dose ranges. The normalized dose response was plotted with log concentrations. The determination of half maximal inhibitory concentration values was done using non-linear regression analysis (Prism). Error bars explain the standard error of the mean percentage (standard error of the mean) for triplicate analysis.

the most challenging issue (Mojibi et al., 2022). Human colon neoplastic disorders represent the third most identified cancer in the United States for both males and females (Jemal et al., 2009). The results obtained from the cytotoxic study demonstrate that the different antiproliferative activities of both *C. oblonga* parts are due to the difference in their chapter three results and discussion 89 polyphenol composition (Costa et al., 2009). These compounds are responsible for the antioxidant and cytotoxic effects of many natural products, as mentioned in previous studies (Fresco et al., 2006). Quince fruit ethyl acetate extracts are rich in flavonoids like apigenin, luteolin, kaempferol, astragalin, and isorhamnetin and this contributes to the cytotoxic activity of human colon cancer cells (Caco-2). The poor inhibition effect of seed extract may be either due to needing a higher concentration to give the complete effect or the cancer cell lines perhaps have low sensitivity to this extract. However, the seeds methanolic extract of Portugal-cultivated *C. oblonga* gives an excellent inhibitory effect on human renal cancer cells (A-498) at about 90%, as presented in previous studies (2). Despite chemotherapy being well-established in the treatment of cancer, it can result in severe side effects, while the use of medicinal plant products produces fewer side effects than chemotherapy (Amaral et al., 2019). Chemical mediators that are targets for flavonoids in fighting colon cancer cells are caspase, nuclear factor kappa B, mitogen-activated protein kinase/p38, matrix metalloproteinase (MMP)-2, MMP-7, and MMP-9, p53, β-catenin, cyclin-dependent kinase (CDK)2 and CDK4, and cyclins A, B, D, and E (Koosha et al., 2016).

Conclusion

The phytochemicals that were identified in the *C. oblonga* seeds and fruit extracts were apigenin, luteolin, kaempferol, astragalin, and isorhamnetin. Those active constituents are believed to be responsible for the antineoplastic activity of the quince extract. Cytotoxic assays were performed on Caco-2 cell lines using both extracts. This revealed that the fruit extract significantly inhibited the growth of the tested cancer cells, while the seed extract showed no effect.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the University of Al-Mustansiriyah, Baghdad, Iraq.

Funding

The paper was extracted from the PhD dissertation of Noor Salman Obaid, approveds by the Department of Pharmacognosy, College of Pharmacy, University of Karbala, Karbala, Iraq.

Authors' contributions

All authors contributed equally to the conception and design of the study, data collection and analysis, interpretation of the results, and drafting of the manuscript. Each author approved the final version of the manuscript for submission.

Conflict of interest

The authors declared no conflict of interest.

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