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First Report on *Cydonia oblonga* Miller Anticancer Potential: Differential Antiproliferative Effect against Human Kidney and Colon Cancer Cells

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The present study reports the phenolic profile and antiproliferative properties of quince (*Cydonia oblonga* Miller) leaf and fruit (pulp, peel, and seed) against human kidney and colon cancer cells. The phenolic profiles of quince methanolic extracts were determined by high-performance liquid chromatography (HPLC)/diode array detector (DAD). 5-*O*-Caffeoylquinic acid was always one of the two major phenolic compounds present in all extracts, except for seed. Our results revealed that quince leaf and fruit extracts exhibited distinctive antiproliferative activities. The extracts from quince leaf showed concentration-dependent growth inhibitory activity toward human colon cancer cells (IC_{50} = 239.7 ± 43.2 μg/mL), while no effect was observed in renal adenocarcinoma cells. Concerning the fruit, seed extracts exhibited no effect on colon cancer cell growth, whereas strong antiproliferative efficiency against renal cancer cells was observed for the highest concentration assayed (500 μg/mL). The antiproliferative activity of pulp and peel extracts was low or absent in the selected range of extract concentrations. This is the first report showing that *C. oblonga* may be useful as a cancer chemopreventive and/or chemotherapeutic agent.

KEYWORDS: ***Cydonia oblonga*** **Miller; quince; phenolic compounds; antiproliferative activity; kidney cancer; colon cancer**

INTRODUCTION

Cancer represents a major public health problem in the United States and many other parts of the world (*1*). Colorectal cancer is currently the third most commonly diagnosed cancer for both men and women in the United States, while kidney cancer ranks seventh among men and eighth among women (*1*). Noteworthy, neither chemotherapy nor radiation has presently a role in the treatment of localized renal cell carcinoma. Surgery is the mainstay of treatment for these patients (*2*). Therefore, the search for new compounds that can safely and effectively block or reverse cancer development remains a priority. In recent years, there has been a substantial increase in studies on the effects of natural plant-derived compounds in cancer prevention and treatment. Polyphenols are nowadays considered potentially important constituents of the human diet for the chemoprevention of cancer (*3*). Suggested mechanisms to explain their antitumoral activities include (i) suppression of nuclear factor-kB (NF-kB) activation, (ii) suppression of activator protein-1 (AP-1) transcription factor activation, (iii) suppression of mitogen-activated protein kinases (MAPKs), (iv) suppression of protein kinases (PKs), namely, PKC, (v) suppression of growth-factor receptor (GFR)-mediated pathways, (vi) cell cycle arrest and induction of apoptosis, (vii) antioxidant and anti-inflammatory effects, and (viii) suppression of angiogenesis (reviewed in refs *4* and *5*).

Studies conducted in the past few years by Silva and coworkers (*6*−*12*) have demonstrated that *Cydonia oblonga* Miller (also known as quince) is a good, safe, and low-cost natural source of different classes of phenolic compounds, such as flavonol and flavone heterosides and, especially, caffeoylquinic acids. These compounds could provide a chemical basis to some health benefits claimed for quince leaf and fruit in folk medicine, namely, in cardiovascular diseases, hemorrhoids, bronchial asthma, and cough (*7*, *10*, *13*). At present, quince fruit is recommended as an important dietary source of health-promoting compounds, because of its chemical composition and antioxidant, antimicrobial, and antiulcerative properties (*6*−*9*, *12*, *14*−*20*). Continuing the research into the biological properties of *C. oblonga*, we recently showed that quince fruit (peel and pulp) and leaf confer protection against oxidative damage of human erythrocytes (*11*, *12*). Moreover, the quince leaf antioxidant effect was similar to that of green tea (*11*). Notwithstanding several well-known biological properties of *C. oblonga*, its anticancer activity is hitherto not known.

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The majority of recent mechanistic studies mainly focus on the anticancer properties of specific pure phenolic compounds, such as quercetin, 5-*O*-caffeoylquinic acid, and catechins, among others (*21*−*26*). Nevertheless, combinations of polyphenols naturally found in fruits and vegetables have been suggested to be most favorable for cancer prevention and their anticarcinogenic effects (*27*). Therefore, the main endeavor of the present study was to investigate the potential human cancer cell antiproliferative activity of *C. oblonga* phytochemicals as found naturally in this plant. For this purpose, methanolic extracts of quince leaf and fruit (pulp, peel, and seed) were prepared, their phenolic profiles were determined by high-performance liquid chromatography (HPLC)/diode array detector (DAD), and the suppression of the proliferation of selected human cancer cells was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) bioassay. The human cancer cells used in this research included the A-498 and 769-P renal and Caco-2 colon carcinomas. As far as we know, this is the first time that human cancer cell antiproliferative properties of *C. oblonga* fruit and leaf extracts have been evaluated.

MATERIALS AND METHODS

Chemicals and Reagents. The standards were obtained from Sigma (St. Louis, MO) and Extrasynthése (Genay, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). MTT and all other chemicals were obtained from Sigma (St. Louis, MO).

Sample Preparation and Extraction. Healthy *C. oblonga* leaves were collected in Carrazeda de Ansiães, northern Portugal, in June 2007 [given that the phenolic total content of leaves is higher in this month (*10*)] and then dried in a stove at 30 ± 2 °C for 5 days (in the dark). Healthy ripe quince fruits were harvested in October 2007 in the same place and separated into pulps, peels, and seeds, and each part was freeze-dried. Lyophilization was carried out using a Labconco 4.5 apparatus (Kansas City, MO).

For methanolic extract preparation, 1.5 g of each dried/lyophilized sample (2.5 g for the seed sample) was thoroughly mixed with methanol (3 × 25 mL and 3 × 10 min) at 40 °C. The methanolic extracts were filtered and concentrated to dryness under reduced pressure. The extraction yields in relation to dry matter were variable as follows: 34, 80, 62, and 12% for leaf, pulp, peel, and seed, respectively. Finally, extracts were redissolved in dimethylsulfoxide (DMSO) to obtain 50 mg/mL stock solutions.

Identification and Quantification of Phenolic Compounds. A total of 20 μL of stock solutions was analyzed using an analytical HPLC unit (Gilson) and a C18 Spherisorb ODS2 column (25.0 × 0.46 cm; 5 μm, particle size) from Waters (Ireland). The solvent system used was a gradient of water/formic acid (19:1) and methanol, as previously described (*11*, *12*). Detection was achieved with a Gilson DAD. Spectral data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 350 nm. Chromatographic data were processed by Unipoint System software from Gilson Medical Electronics (Villiers le Bel, France). The compounds in each sample were identified by comparing their retention times and UV−vis spectra in the 200−400 nm range to the library of spectra previously compiled by the authors (*10*−*12*). Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3-*O*- and 4-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid. Kaempferol-3-*O*-glycoside was quantified as kaempferol-3-*O*-glucoside, and kaempferol glycoside acylated with *p*-coumaric acid was quantified as kaempferol-3-*O*-rutinoside. Quercetin glycoside acylated with *p*-coumaric acid was quantified as quercetin-3-*O*-rutinoside. Luteolin, apigenin, and chrysoeriol derivatives were quantified as luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, and chrysoeriol, respectively. The other compounds were quantified as themselves.

Cell Proliferation Assay. Human renal epithelial cancer cells A-498 (ATCC HTB-44) and 769-P (ATCC CRL-1933) were cultured in RPMI 1640 medium (GlutaMax Media, Gibco BRL Life Technologies) with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) containing 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen), 100 units/mL penicillin (Gibco, Invitrogen), and 100 μg/mL streptomycin (Gibco, Invitrogen). Human colon cancer Caco-2 (ATCC HTB-37) cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1 (Gibco BRL Life Technologies) and supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (Gibco, Invitrogen). For the assay, cells were grown in T-75 culture flasks in a humidified incubator at 37 °C under a 5% CO_2/95% air mixture. After trypsin detachment, A-498, 769-P, and Caco-2 cells were counted, subcultured at 5×10^3 cells per well of a 96-well microplate, and incubated to allow for cell attachment. After 24 h of incubation, the cells were treated with different concentrations of quince leaf and fruit (pulp, peel, and seed) extracts. Stock solutions (50 mg of extract/mL in DMSO) were accurately diluted in medium to obtain final concentrations of 31.25, 62.5, 125, 250, and 500 μg of extract/mL. From these serial dilutions, the DMSO final concentration was never higher than 1% and the same concentration was used for solvent−control wells. The plates were incubated for 48 h under the same conditions. At the end of the exposure time, the antiproliferative activity of quince extracts on the tumor cells was measured by evaluating cell viability using the MTT colorimetric assay, as previously described by Carmichael et al. (*28*), with some modifications. Briefly, after exposure to the extracts, the medium was removed and the cells were washed once with fresh culture medium. Subsequently, the cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 4 h with 200 μL of 0.5 mg/mL MTT (final concentration). MTT solution was then removed, and intracellular MTT−formazan crystals were solubilized in 100 μL of dimethyl sulfoxide. The absorbance was measured at 550 nm in a microplate reader. Cell viability was measured as the percentage of absorbance compared to the control. The 50% inhibitory concentration (IC_{50}) values, defined as the amount of extract that inhibits 50% of cell growth, were calculated from concentration−response curves following a 48 h exposure time. Experiments were performed in triplicate and repeated at least 3 times.

Statistical Analysis. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 16.0) for Windows. Multiple comparisons between more than two groups were performed by one-way analysis of variation (ANOVA) supplemented with Tukey's honestly significant difference (HSD) post-hoc test. Significance was accepted at p lower than 0.05.

RESULTS AND DISCUSSION

Phenolic Profile of Quince Extracts. Methanolic extracts of quince parts contain significant amounts of polyphenolic compounds (**Table 1**). Total phenolic content decreased in the following order: leaf > peel > pulp > seed (27.96, 7.41, 1.17, and 0.52 g/kg of methanolic extract, respectively).

Pulp extract presented a chemical profile composed of only two phenolic compounds (**Table 1**): 3-*O*- and 5-*O*-caffeoylquinic acids.

With regard to the peel extract, eight phenolics were identified (**Table 1**): the two hydroxycinnamic acids found in pulps, plus 4-*O*-caffeoylquinic acid, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, and three partially identified compounds (a kaempferol glycoside, a quercetin glycoside acylated with *p*-coumaric acid, and a kaempferol glycoside also acylated with *p*-coumaric acid).

The seed extract presented a characteristic phenolic profile, composed of the same caffeoylquinic acids found in pulp and several *C*-glycosyl flavones: lucenin-2 (6,8-di-*C*-glucosyl luteolin), vicenin-2 (6,8-di-*C*-glucosyl apigenin), stellarin-2 (6,8-di-*C*-glucosyl chrysoeriol), and schaftoside (6-*C*-glucosyl-8-*C*-arabinosyl apigenin) and two partially identified compounds (6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol and 6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol) (**Table 1**).

Leaf extract was characterized by the presence of seven phenolics (**Table 1**): 3-*O*-, 4-*O*-, and 5-*O*-caffeoylquinic acids, 3,5-*O*-dicaffeoylquinic acid, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glycoside, and kaempferol-3-*O*-rutinoside.

Table 1. Phenolic Composition of Quince Pulp, Peel, Seed, and Leaf Methanolic Extract[a]

phenolic compound	pulp	peel	seed	leaf
3-*O*-caffeoylquinic acid	0.50 ± 0.01	1.14 ± 0.09	0.01 ± 0.00	5.54 ± 0.38
4-*O*-caffeoylquinic acid	nd	0.18 ± 0.01	nd	0.64 ± 0.05
5-*O*-caffeoylquinic acid	0.67 ± 0.01	1.65 ± 0.11	0.06 ± 0.00	10.67 ± 0.67
lucenin-2	nd	nd	0.03 ± 0.00	nd
vicenin-2	nd	nd	0.07 ± 0.00	nd
stellarin-2	nd	nd	0.15 ± 0.01	nd
schaftoside	nd	nd	0.05 ± 0.00	nd
6-*C*-pentosyl-8-*C*-glucosyl chrysoeriol	nd	nd	0.10 ± 0.01	nd
6-*C*-glucosyl-8-*C*-pentosyl chrysoeriol	nd	nd	0.05 ± 0.00	nd
3,5-*O*-dicaffeoylquinic acid	nd	nd	nd	2.22 ± 0.14
quercetin-3-*O*-rutinoside	nd	3.29 ± 0.12	nd	4.90 ± 0.26
kaempferol-3-*O*-glycoside	nd	0.14 ± 0.00	nd	1.52 ± 0.05
kaempferol-3-*O*-glucoside	nd	0.55 ± 0.01	nd	nd
kaempferol-3-*O*-rutinoside	nd	nd	nd	2.47 ± 0.09
quercetin glycosides acylated with *p*-coumaric acid	nd	0.22 ± 0.01	nd	nd
kaempferol glycosides acylated with *p*-coumaric acid	nd	0.24 ± 0.02	nd	nd
$\sum$	1.17	7.41	0.52	27.96

[a] Values are expressed as mean ± standard deviation of three assays for each sample (g/kg of methanolic extract). Abbreviations: nd, not detected; $\sum$, sum of the determined phenolics).

The main phenolic compounds were 5-*O*-caffeoylquinic acid for pulp and leaf (57 and 38%, respectively), quercetin-3-*O*-rutinoside for peel (44%), and stellarin-2 for seed (29%). In fact, 5-*O*-caffeoylquinic acid was always one of the two major phenolic compounds present in *C. oblonga* leaf, pulp, and peel extracts.

Human Cancer Cell Antiproliferative Activity of Quince Extracts. Kidney and colon cancers are among the most frequent cancer types (*1*). Therefore, in the present study, quince leaf and fruit methanolic extracts were evaluated for their ability to inhibit the growth of human renal (A-498 and 769-P) and colon (Caco-2) cancer cell lines. Panels **A**−**C** of **Figure 1** present the concentration effectiveness of quince extracts on the viability of A-498, 769-P, and Caco-2 cells. Five different concentrations of each extract (31.25, 62.5, 125, 250, and 500 μg/mL) were tested. The extracts from quince leaf showed concentration-dependent growth inhibitory activity toward human colon cancer cells (**Figure 1C**), while no effect was observed in renal adenocarcinoma cells (panels **A** and **B** of **Figure 1**). An IC_{50} value of 239.7 ± 43.2 μg/mL was obtained for leaf extract.

Concerning the fruit, seed extracts exhibited no effect on colon cancer cell growth (**Figure 1C**), whereas the highest extract concentration was effective against both A-498 (**Figure 1A**) and 769-P (**Figure 1B**) cells. The seed extract exhibited 91 and 84% cell growth inhibition at a concentration level of 500 μg/mL against A-498 and 769-P cells, respectively. Of note, renal cell carcinoma is highly resistant to current chemotherapeutic regimens, and studies conducted on cell cultures have shown to be a barely responsive cancer cell line with few natural extracts showing significant growth inhibition effects toward these cells (*29*). Therefore, these results strongly suggest quince seed extract as a promising anticancer agent for kidney cancer. However, contrary to quince leaf, this effect did not seem to be dependent upon the extract concentration, and therefore, the IC_{50} value for seed extract may be somewhere in a 250−500 μg/mL range.

The antiproliferative activity of pulp and peel extracts was low or absent in the tested range of extract concentrations. The highest concentration of pulp extract (500 μg/mL) significantly reduced the proliferation of A-498 (**Figure 1A**) and Caco-2 cells (**Figure 1C**) in about 34 and 18%, respectively, but no significant effect was observed with the peel extract. The IC_{50} values of pulp and peel extracts toward A-498, 769-P, and Caco-2 cells could not be determined at the concentrations tested in this experiment because cell proliferation was not inhibited to 50% (IC_{50} > 500 μg/mL).

Recent studies conducted in both cell cultures and animal models seem to indicate that polyphenols are the main phytochemicals with antioxidant and antiproliferative properties of higher plants (*6*, *7*, *14*, *17*, *30*−*34*). Inhibition of growth and induction of apoptosis by caffeoylquinic acid derivatives, such as the 5-*O*-caffeoylquinic acid found in all quince parts, have been shown in several human cancer cell lines (*35*). The molecular targets of quince caffeoylquinic acids for prevention and therapy of cancer include inhibition of growth factors, such as interferon-γ and interleukin-2, extracellular signal-regulated protein kinase1/2, serine/threonine protein kinase, NF-kB, and inducible nitric oxide synthase (*36*). These compounds are also well-known by their antioxidation properties, preventing oxidative damage of cellular DNA, lipids, and proteins (*11*, *12*, *37*). In addition, the antioxidant, anti-inflammatory, and anticancer effects of flavonols, such as quercetin and kaempferol, and flavones, such as luteolin and apigenin, have also been reported and reviewed (*4*, *21*−*24*, *32*, *38*). These compounds and their derivatives exhibit antioxidant and free-radical-scavenging capacities, induce cell cycle arrest, promote selective apoptosis within tumors via intrinsic and extrinsic signaling pathways, and suppress tumor growth by blocking angiogenesis (*4*, *21*, *23*−*25*). A correlation between the structural oxidation state of flavonoids and the position, number, and nature of substituents and their antiproliferative effects has been suggested (*38*). The hydroxylation pattern of the B ring of the flavones and flavonols, such as luteolin and quercetin, seems to critically influence their activities, especially the inhibition of protein kinase activity and antiproliferation (*21*). In fact, in comparison to other flavonoids, luteolin and quercetin are usually among the most effective ones (*26*, *31*). In accordance, Alesiani et al. (*39*) reported the antiproliferative activity of several phytochemicals isolated from quince peels, namely, caffeoylquinic acids, quercetin, and rutin, on murine B16-F1 melanoma cells; among these, quercetin was shown to be the most effective phenolic compound.

No correlation was found in the present study between the total content of polyphenols in the quince extracts and inhibition of cancer cell proliferation, despite the above reports describing the antiproliferative activity of single pure polyphenolic compounds. In view of the number of phytochemicals and the complexity of the mechanisms proposed for their chemopreventive properties, this might be due to the unique combination of constituents (with complementary activities) present in each extract.

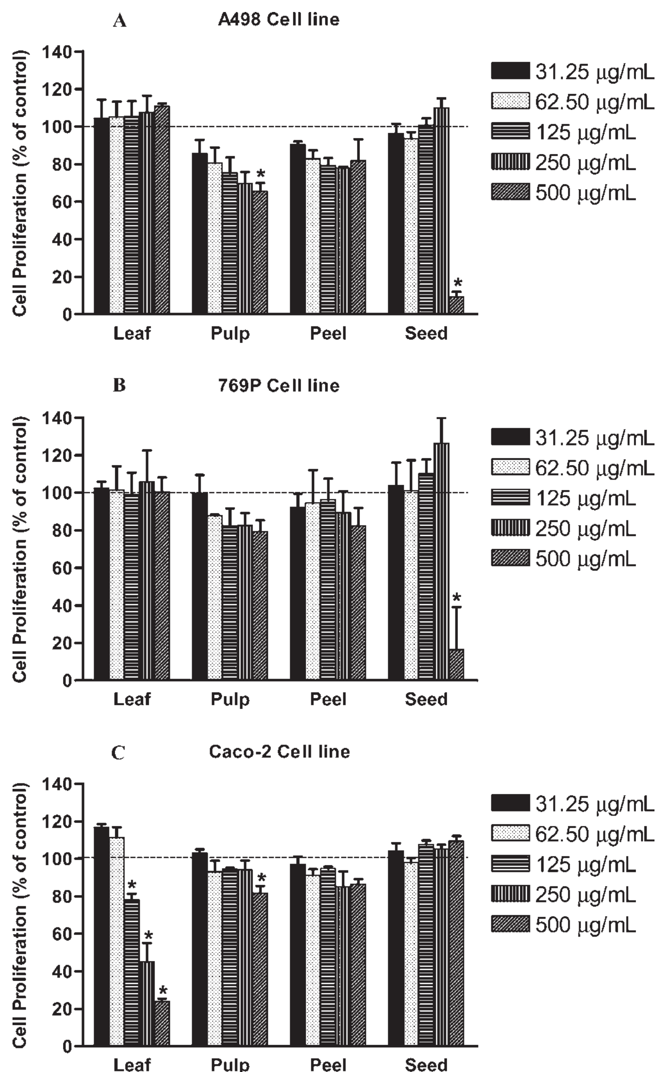

Figure 1. Inhibition of proliferation of A-498, 769-P, and Caco-2 human cancer cell lines by methanolic extracts of quince leaf, pulp, peel, and seed. Cells were exposed to five concentrations of extracts (31.25, 62.5, 125, 250, and 500 μg/mL) for 48 h. Values are presented as mean $\pm$ standard deviation (SD) of three independent experiments, performed in triplicate. ($*$) $p < 0.05$, as compared to the control.

Additionally, our results show a considerable difference in the sensitivity of human cancer cells to quince extracts, probably because of the fact that these extracts are rich in different classes of phenolic compounds, such as hydroxycinnamic acid derivatives and flavonol or flavone heterosides. In fact, leaves contain the highest content of polyphenols between quince parts, mainly caffeoylquinic acids and quercetin and kaempferol derivatives, that are most likely responsible for the remarkable antiproliferative activity of quince leaf extract against colon cancer cells. In agreement, our previous studies indicate that leaves exhibit the strongest antioxidant effect among all quince parts, which may prevent or slow down oxidative stress-induced damage leading to cancer (*11*, *12*). On the other hand, the inhibitory effect in renal cancer cell growth observed for seed extract may be explained by its distinctive phenolic composition, especially to the presence of apigenin, luteolin, and chrysoeriol *C*-glycosides. Moreover, it was suggested that the hemolytic activity and foaming properties of seed extracts are probably due to the presence of saponins (*12*), but these secondary metabolites have not yet been investigated in this matrix. As polyphenols, saponins are well-known by their anticancer activities (*31*).

In conclusion, the present study demonstrates for the first time that quince leaf and fruit (mostly the seed) present effective human cancer cell antiproliferative activity against colon and renal cancers, respectively. This differential antiproliferative effect is most likely related to the distinctive structural classes of *C. oblonga* constituents found in leaves and seeds. Our results suggest *C. oblonga* species as a promising agent for cancer prevention and/or treatment and will certainly encourage future studies to increase our knowledge on the anticancer potential of this plant.

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