Anti-oxidant and Selective Anti-proliferative Effects of the Total Cornicabra Olive Polyphenols on Human Gastric MKN45 Cells

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Background: According to the epidemiological studies, consuming olive products can decrease the incidence of the different types of cancers mostly due to the high anti-oxidant properties of their polyphenolic compounds.

Objectives: To evaluate the anti-oxidant and anti-proliferative potentials of the olive fruits total polyphenols on the gastric adenocarcinoma MKN45 cells in comparison to the normal Hu02 cells.

Materials and Methods: The total phenolic content of the olive fruits and radical scavenging activity were determined by Folin and 2,2-diphenyl-1-picrylhydrazyl (DPPH) tests respectively. MTT assay was performed for the evaluation of the cell viability. Intracellular reactive oxygen species (ROS) level was measured using DCFH-DA. Statistical analysis was performed using SPSS 16 statistical software.

Results: Treatment of the MKN45 cells with the phenolic compounds extracted from olive fruits decreased growth and viability of the cells in a dose- and time-dependent manner. In addition, treatment of the MKN45 cells with a combination of the phenolic compounds extracts and cytarabine further decreased cell compared to monotherapy of the cells with each compound alone. Mechanistically, we showed that the anti-cancer effects of the olive polyphenols in the MKN45 cells are mediated through depletion of ROS. Similarly, polyphenolic extracts were found to decrease ROS level in the normal cells at the concentrations of 500 and 1000 μg.ML 
 and short treatment times (6 h), but the viability of these cells did not significantly change. At high concentrations (2000 μg/mL) of the polyphenolic extracts or at longer times of incubation (12 h), however, both ROS levels and the viability of the cells were significantly decreased in the normal cells.

Conclusions: The olive fruits polyphenolic extract modulates ROS levels and selectively targets cancerous cells at low concentrations. Also, the effects of cytarabine could be potentiated by the olive fruits polyphenols. Thus, for a combined protocol of cancer cell therapy, olive fruit polyphenolic compound could be proposed as a proper candidate.

Keywords: Cytarabine; Olea; Sunphenon; Reactive Oxygen Species; Stomach Neoplasms

1. Background
Olive oil obtained from the olive fruit is a source of polyphenolic compounds, a mixture of organic compounds that olive plant produces in order to confront biotic/abiotic stresses which the plant was continuously exposed in the during of growth and development (1). Recent studies have shown that consuming virgin olive oil with a higher ratio of polyphenols reduces the oxidative damage (2) and is capable of improving various physiological features in the individuals who daily consume virgin olive oil in their diet. Based on epidemiological studies, consuming olive products is associated with the low incidence of a variety of cancers, including leukemia, colorectal carcinoma, and breast cancers (3).

Recent reports indicate that the chemo-preventive ability of the olive oils is mostly related to their high anti-oxidant properties. It has been recently suggested that the polyphenolic compounds can be used in adjuvant therapy approaches for the cancer treatment (4, 5). For example, Menendez et al. showed that olive oil
polyphenols not only can be used in adjuvant therapy for the killing of tumor cells but also can be utilized for designing a new generation of the drugs (6). The beneficial effects of the olive oil in cancer are mostly through a synergy of all its major and minor components that can generate a variety of cell responses involved in the cancer prevention and treatment (7, 8). In fact, polyphenols modulate oxidative stress in cancer cells through modulation of signal transduction and the expression of specific genes related to the cell proliferation and cell death (9, 10). As an evidence, polyphenol compounds trigger apoptotic programmed cell death pathways in human gastric carcinoma cells via manipulation of ROS content of the cancerous cells. Conceptually, the ROS modulating effects of the polyphenols can increase or decrease in basal ROS levels of the cells, proposing a new therapeutic strategy based on pro-oxidant or anti-oxidant therapy, respectively. Due to the difference in the basic ROS levels and oxidative stress status between normal and cancerous cells, these therapeutic approaches are used for the selective targeting of cancerous cells. For example, it has recently been reported that a controlled manipulation of the ROS can selectively target leukemia cells but not normal cells (11). Also, it has been reported that normal cells are less sensitive to the polyphenols compared to cancer cells (12, 13).

The incidence of cancer and its mortality rate is rising worldwide (14), and among cancers, gastric cancer is one of the leading cause of cancer death (15, 16). A positive growing trend of cancer caused death from stomach cancer (the most common type of cancer in men) toward gastric cancer has been recently reported in several parts of the world (17). Obviously, any effort in finding new anti-cancer drugs and strategies has a high priority in the cancer research.

2. Objectives
This study aimed to evaluate anti-oxidant potential of the polyphenolic compound extracted from olive fruits and to study its effects on the growth and viability of the human gastric cancer cell line MKN45 in comparison to the normal Hu02 cells.

3. Materials and Methods

3.1. Sampling and Preparation of the Total Polyphenol Extract (TPE)
The olive fruits of Cornicabra variety were collected in the autumn of 2013. Cornicabra is a Mediterranean commercial olive variety that is known as a rich source of polyphenolic compounds (18). The collected fruit samples were immediately frozen in the liquid nitrogen and were transferred to the laboratory. The pulps of the fruits were powdered through grinding in the liquid nitrogen; 3 gr of which was dissolved in 12 mL of methanol (methanol/H2O, 80%) and then centrifuged (2500 rpm) at 4 °C for 20 min. The supernatant was collected as the total/methanolic extract and was freeze-dried and stored at -20 °C before usage. The supernatant was collected and TPE (olive/methanol extract) were obtained and immediately used for Folin and DPPH assessments.

3.2. Determination of the Total Phenolic Content (TPC)
The total phenolic content was measured according to the modified Folin-Ciocalteu’s reagent method (19). Briefly, the primary methanolic extract was 10 times more diluted using methanol and 30 μL of which was mixed with 100 μL of the Folin-Ciocalteu’s reagent. After mixing for 1 min, 300 μL of 20% (w/v) sodium carbonate solution was added and the mixed solution was kept for 2 h in the dark at room temperature. The absorbance of the reaction mixture was measured at 765 nm. The result was expressed as mg of Gallic acid equivalent per ml of the fresh weight (mg GAE.mL fresh weight) for obtaining calibration curve of Gallic acid.

3.3. Determination of Radical Scavenging Activity (RSA) of TPE
2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay of the sample was carried out according to the standard method with some modifications (20). Several concentrations of each sample were tested and 10-100% of the free radical scavenging activity was determined. At first, 2.7 mL methanol was mixed with 0.3 mL of 0.10 mM DPPH methanolic solution and then 20 μL of the extract was added. After an incubation period for 30 min at room temperature and dark condition, the absorbance was determined compared to a blank sample at 517 nm (21). The percentage of DPPH inhibition was calculated as follow:

\[
\text{DPPH scavenging }\% = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100
\]

The pure DPPH solution was used as a control group and methanol was used for the baseline correction. The extract concentration that provided 50% inhibition (EC50 μg.mL⁻¹) was calculated from the log-dose inhibition curve regression equation. EC50 values are referred to the lower concentration of the compounds required for the 50% of the anti-oxidant activity.

3.4. Cell Culture and Treatment of Cells
For cell studies, the lyophilized extract was dissolved in PBS and sterilized by filtration (0.22 mm). The human gastric adenocarcinoma cell line (MKN45) and human fibroblast cell line (Hu02) was obtained from Iranian Biological Resources Center (IBRC) Cell Bank, Tehran, Iran. MKN45 cells were routinely grown in DMEM (Gibco, USA) containing 10% FBS, 50 μL·mL⁻¹ penicillin and 50 μg·mL⁻¹ streptomycin in a humidified incubator at 37 °C supplied with 5% CO₂. The harvested cells were seeded into 96-well plates (1×10⁴ cell.well⁻¹). The sterilized extracts were used for the treatment of the cells. After 24 h of incubation, the cells were treated with
the different concentration of the olive fruit extracts, Cytarabine (500, 1000, and 2000 μg.mL\(^{-1}\)) separately or in combination (i.e., different combination of the concentrations: olive extract + Cytarabine (250 + 2.50 μg.mL\(^{-1}\)), olive extract + Cytarabine(500 + 500 μg.mL\(^{-1}\)), and olive extract + Cytarabine (1000 + 1000 μg.mL\(^{-1}\)) were used and treatment was carried out for 24, 48, and 72 h.

3.5. Cell Viability Analysis
The effect of the olive extract on cell proliferation was evaluated by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay with some modifications as described by Scudiero et al. 1988. MTT assay and growth inhibition were assessed 24 h, 48 h, and 72 h after the addition of the extracts and results were reported as the percentage of cell viability of the non-treated cells compared to the control group. Briefly, control and treated cells were collected at different time intervals, then 100 μL MTT (100 μg.mL\(^{-1}\)) was added to each well of 96-well plates. Plates were then incubated for 4 h at 37 °C, then the whole medium was removed and 100 μL DMSO was added for solving the formazan crystals. The difference in the purple color between samples was measured at 570 nm using a plate reader (BioTeck, USA). For each treatment, cell viability was calculated using the below formula:

\[
\text{Viable cells (%) = } \left( \frac{\text{OD}_{\text{treated cells}}}{\text{OD}_{\text{control}}} \right) \times 100
\]

3.6. Measurement of the Intracellular ROS Generation
The intracellular reactive oxygen species (ROS) level was measured using a cell-permeable fluorescent probe, 2',7'-dichlorofluorescein di-acetate (DCFH-DA) (22). Upon oxidative stress, this probe is cleaved by the nonspecific esterases and forms DCF with the high fluorescent intensity (23). Briefly, cells were seeded into 96-well culture plates and treated with the different concentrations of polyphenol extracts for different time intervals (24). The extracted polyphenol protective capacity against oxidative stress was evaluated through incubation of the cells with a combination of extract plus H2O2. All treatments were considered for 6 h, 12 h and 24 h. Then, the cells were incubated with DCFH-DA (15 μM in PBS) for 40 min at 37 °C in 5% CO2. The fluorescence intensity was quantified at 485/535 nm and compared with the fluorescence of the non-treated cells. The corresponding absorbance values were obtained by applying a fluorescent ELISA reader (Synergy H4 hybrid reader – Biotech). The percentage of the intracellular ROS level was calculated as the ratio of the fluorescence of treated cells/fluorescence of non-treated cells, respectively.

3.7. Statistical Analysis
The statistical analysis was performed by One-way ANOVA (P < 0.05) using SPSS 16 statistical software. The student t-test was used for identification of the significant differences. All the analyses were done in triplicates, and each performed in three independent experiments.

4. Results
4.1. In vitro Anti-Oxidant Activity of the Total Phenol Content (TPC)
At first step of this study, we extracted the total polyphenol from the Cornicabra olive variety to evaluate its anti-oxidant activity. As indicated in Table 1, TPC was 8.4 ± 0.03 mg.g\(^{-1}\) GAE/FWP in the fruit samples which harvested at 150 days after flowering (DAF). In this condition, the percentage of the free radical inhibition was 31.26 ± 0.04 and RSA was calculated as 2.5 ± 0.04 mg.mL\(^{-1}\). According to the results presented in Table 1, a direct relationship between the phenolic content and anti-oxidant capacity of the examined olive extract can be observed.

| Cultivar    | TPC (mg.g\(^{-1}\)) | % of DPPH scavenging | RSA (mg.g\(^{-1}\)) |
|-------------|---------------------|-----------------------|--------------------|
| Cornicabra  | 8.4 ± 0.03          | 31.26 ± 0.04          | 2.5 ± 0.04         |

4.2. Anti-Proliferative Effects of the Olive Polyphenols, Alone or in Combination with Chemotherapy Agent, on the MKN45 and Hu02 Cells
At next step, the anti-proliferative effects of the polyphenolic extracts from the olive fruits were studied on MKN45 cells (Fig. 1A-C). The results showed a dose- and time-dependent decrease in the viability of cancerous MKN45 cells (Fig. 1A-C). For example, cell viability was reduced by 65, 64 and 58 % after 24 h treatment with the concentrations of 500, 1000, and 2000 μg.mL\(^{-1}\) of the polyphenolic extract, respectively, in MKN45 cells. Cytarabine, a chemotherapeutic drug, at concentrations of 500, 1000, and 2000 μg.mL\(^{-1}\) inhibited the growth of MKN45 cells by 87, 82, and 71%, respectively. Interestingly, a combination of therapy with olive extract and drug (Fig. 1A-C) further decreased the viability of the cells by 60%, 55%, and 54% for 500, 1000, and 2000 μg.mL\(^{-1}\) concentrations after 24 h, respectively. These anti-cancer effects of the extract (alone or in combination with the drug) were further increased after 48h and 72 h (Fig. 1B and 1C). To investigate the effects of extract on normal cells, we selected human foreskin fibroblast Hu02 cells. Notably, low concentrations (500 and 1000 μg.mL\(^{-1}\)) of the olive
extract, Cytarabine and their combinations showed no significant effect on the viability of the normal Hu02 cells after 24 and 48 h (Fig. 1D and E). A marginal decrease in the viability of the Hu02 was observed at 1000 μg.mL\(^{-1}\) after a longer treatment time (72 h), suggesting that anti-cancer effects of low concentration

of the olive extracts can be used for a selective targeting of the cancerous cells (Fig. 1F). However, similar to MKN45 cells, the high concentrations of extract (2000 μg.mL\(^{-1}\)) drug and their combinations showed a potent cell death induction in the normal cells (Fig. 1D-F).

![Figure 1](image)

**Figure 1.** The effects of the total olive fruits polyphenolic extract (Corni) and its combination therapy with cytarabine on the viability of MKN45 (A, B, and C) and Hu02 (D, E and F) cells. The cells were treated with different concentrations of corni (500, 1000 and 2000 μg.mL\(^{-1}\)) and cytarabine (500, 1000 and 2000 μg.mL\(^{-1}\)) as well as their combination therapies, then viability was determined after 24 h (A, D), 48 h (B, E), and 72 h (C, F) in both Cells. Results were reported as percent of control (%) and expressed as average ± S.E. differences (\(p < 0.05\)) were considered statistically significant in comparison with the controls (*)

4.3. Olive Fruits Extract Concentration and MKN45 Cell Death Morphological Signature

To get more insight into the effects of olive extract, we studied the morphology of MKN45 cells following treatment with different concentrations of the polyphenol extracts (Fig. 2).

As can be observed in Fig 2 the typical signs of cell death (i.e. cell debris, aggregation, and shrinking) were detected in a concentration-dependent manner (Fig. 2A-F). In facts, the morphological changes corresponding to the cell death were clearly observed after incubation with 1000 and 2000 μg.mL\(^{-1}\) concentrations of the extract (Fig. 2E and F). These results (Fig. 2) are in full agreement with the viability results observed in Figure 1.

![Figure 2](image)

**Figure 2.** The morphological study of the MKN45 cells. non-treated group (A) and treated with 100 (B), 200 (C), 500 (D), 1000 (E) and 2000 (F) μg.mL\(^{-1}\) of corncabra olive extracts after 24 h. Arrows showed apoptotic cell death morphology. Scale bar represents 100 μm in an invert light microscopy image.
4.4. Effects of Olive Fruits Polyphenols, Alone or in Combination with Chemotherapy on MKN45 and Hu02 Cells ROS Content

Since anti-cancer effects of the olive extracts are mediated through modulation of the oxidative stress status of the cells, we were also interested to study the effects of our system on ROS content of both normal Hu02 and cancerous MKN45 cells. As indicated in Figure 3 ROS generation was assayed at various concentrations of the extracts and different times (Fig. 3). Our results revealed that a significant decrease in the basic ROS level of the cancerous MKN45 cells was started after 6 h treatment with polyphenolic extract followed by a more reduction after 12 h (Fig. 3A and B). The effects of the extract on ROS level were different in the normal cells (Fig. 3C and D). While treatment of the Hu02 cells with different concentrations of the extract decreased ROS level after 6 h, the total content of ROS started to increase after 12 h (Fig. 3C and D). To further study the anti-oxidant potential of the extract, we pretreated the cells with H$_2$O$_2$ and evaluated the effects of polyphenols on this strong pro-oxidant system. H$_2$O$_2$ at different concentrations (25, 50 and 100 mM) showed strong ROS production and pro-oxidant effects in both cells (Fig. 3). Our results show that combination of olive extract substantially decreases ROS production by H$_2$O$_2$ in both cells. This effect was observed for all concentrations after 6 and 12 h incubation in both cells (Fig. 3). The effects of the extract in inhibiting the pro-oxidant potentials of H$_2$O$_2$ were higher in Hu02 cells than MKN45 cells.

5. Discussion

Polyphenols are important classes of anti-oxidants in the olive oil (25). It has been evidenced that polyphenols of the olives act as anti-inflammatory components and can prevent cancer progression (26). Recent evidence showed that permanent of the olive oil consumption is associated with a lower risk of cancer development, especially in gastrointestinal cancers. Here, we reported for the first time that polyphenolic extracts of the olive fruits from the Cornicabra variety (this variety is cultivated in some region of Iran) can selectively target cancerous cells at both low concentrations and short treatment times. The morphological studies showed signs of cell death following treatment with different concentrations of the olive extract in cancerous cells (Fig. 2), while normal cells could survive at low concentrations treatment of the extracts (Fig. 1). Whether the mode of induced cell death is through apoptosis or other types of programmed cell death might be involved in this process need more investigation.

From a mechanistic viewpoint, it seems that Cornicabra olive extract manipulates the redox potential of the cells (Fig. 3). In fact, our used extracts have dramatically decreased ROS levels in MKN45 cells after 6 h and 12 h...
These anti-oxidant effects of the extract were observed in the normal Hu02 cell (Fig. 3C and D), albeit at short times (6 h). After longer treatment times (12 h), the ROS level started to increase to the basic levels (500 and 1000 µg/mL) or even more (2000 µg/mL). These different effects of the olive extract on ROS levels of the normal and cancerous cells can be related to the inherent differences between basic ROS levels and anti-oxidant defense systems of both cells (27, 28). In fact, it has been suggested that compared to the normal cells, cancerous cells are under a chronic oxidative stress and are more susceptible to the ROS manipulating strategies than normal cells (29, 30). It seems that anti-oxidative effects of the polyphenols can modulate oxidative stress-responsive genes, leading to the anti-cancer effects. In this condition, however, normal cells can survive because they have normally lower levels of ROS and harbor intact oxidative defense system (31). Recent reports indicated that treatment of cancerous cells with polyphenolic compounds of the olive oil leads to cell ROS modulation and consequently cell death (32, 33). Recently, it has been reported that cell proliferation is inhibited by some olive polyphenols in human HL60 cell line and colon cancer lines via ROS manipulation (32, 34).

Combination therapies with natural polyphenols and the known chemotherapeutic drugs could improve cancer treatment options. Interestingly, combining polyphenols with the conventional therapies might lead to overcoming drug resistance and reducing the side effects of the standard anti-cancer therapies. For instance, several studies have shown that a combination of resveratrol with quercetin or Curcumin has increased the growth inhibition effects compared with each monotherapy in oral squamous carcinoma and colon cancers, respectively (35). Menendez et al. have reported that the polyphenols in the extra virgin olive oil (EVOO) can diminish the acquired resistance to trastuzumab in HER2-overexpressing breast cancer cell lines through activation of the signaling pathways, inducing cell death, and cell cycle arrest in different tumor cell lines as well as their anti-oxidant and anti-inflammatory abilities (36, 37). In line with the above reports, the results of this study show a higher anti-cancer effect for a combination of extract and cytarabine in comparison with each therapy alone. These results are in agreement with the recent reports concerning the additive effects of the extract of the different plants in growth inhibition of the human cancer models (38-40). Polyphenols are double-edged swords that show either beneficial effects at physiologic (low) doses or deleterious effects at pathologic (high) doses (41, 42).

Our results showed that the treatment with extract decreased the pro-oxidant potential of H2O2 in both cancer and normal cell lines as determined by measuring ROS production (Fig. 3). These results fully confirm the anti-oxidant potential of our used extract in the cell culture system and suggest that increased levels of ROS can be scavenged by phenolic compounds. However, it should be mentioned that polyphenols can either scavenge ROS or paradoxically generate additional amounts of ROS to inhibit cancer cells proliferation. It has been observed that the low and high concentrations of the same polyphenolic compound are responsible for anti-oxidant and pro-oxidant activities, respectively (26, 42-45). Moreover, it should be considered that elevated levels of ROS in the cells might be a defensive mechanism to cope with the stress resulted from high dosage administration of the phenol extracts. Thus, further studies are required to elucidate the underlying molecular mechanisms of the olive extracts.

6. Conclusions

Cornicabra olive extract presents a potent inhibitory effect on ROS level and viability of the cancerous MKN45 cells. Accordingly, its combination with cytarabine could sensitize cancerous cells to the chemotherapy. Although olive extract manipulated ROS levels in Hu02 cells, the viability of these normal cells did not significantly change at low concentrations and short treatment times. Thus, Cornicabra polyphenols can selectively target cancerous cells and could be proposed as a candidate for combination therapy of cancer.

Acknowledgments

This work has been partially supported by the Center for International Scientific Studies & Collaboration (CISSC), National Institute of Genetic Engineering and Biotechnology (NIGEB) and Stem Cell Technology Research Center of Iran.

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