Extraction of Phenolic Compounds from Olive Leaf Extracts and Their Effect on Proliferation of Human Carcinoma Cell Lines

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Abstract

The aim of this work was to evaluate the effect of different olive leaf extracts (OLE) from different leaf growing stages on human carcinoma cell lines. OLE were tested in human carcinoma cell lines *in vitro* and cells were plated in 96-microtiter culture plates for each OLE concentration. Fresh (F) and freeze-dried (FD) leaves exhibited phenolic compounds in the range of 2.09 ± 0.10 to 8.44 ± 0.64 and 7.72 ± 0.56 to 24.65 ± 1.9 mg gallic acid equivalents/g leaves, respectively. OLE from several Portuguese olive tree cultivars were found to inhibit the growth of human carcinoma cell lines in a range of 2.09 - 8.44 μg phenolic compound/well (209 - 844 μg/ml) and 0.07 - 2.40 μg phenolic compounds/well (7 - 240 μg/ml) for fresh and freeze-dried leaves, respectively. Young (Y) leaves have revealed the highest cell growth inhibition ranging from about 95% for Cobrançosa, followed by 90% for Cobrançosa, 90% for Arbequina and 75% for Arbequina for cell lines A549, HeLa, A431 and OE21, respectively. The lowest cell growth inhibition (35%) was observed for Galega (Y) leaf extract on cell line A549. However, FD samples exhibited a distinctive pattern since cell growth inhibition was highest at highest extract dilution tested, for A431 (Galega Y) followed by A549 (Cobrançosa Y) with cell inhibition of 75% and 70%, respectively. The data presented in this work strongly suggest that OLEs inhibit the growth of human carcinoma cell lines.

Keywords

Olive Leaf Extracts, Anti-Proliferation of Human Carcinoma Cell Lines, Freeze-Dried Leaves, Phenolic Compounds, Younger and Older Olive Leaves, Portuguese Olive Cultivars

1. Introduction

Cancer is widely known nowadays as the disease of the century due to the number of growing cases worldwide but also to the amount of research dedicated to this field and its increasing success rate [1].

Although the efficiency of chemotherapy for the majority of cancers has improved over the last three decades, they still exhibit high toxic effects that are responsible for severe reduction in quality of life, causing problems in clinical medicine. Therefore, it is important to develop novel potent, but low toxic anti-cancer agents, including natural products [2]. In recent years, Middle East herbal medicine has been of great interest for research workers regarding different strategies for cancer treatment [3] [4]. Traditional Chinese Medicine (TCM) has been using herbal medicinal plants for treatment of diseases for many centuries [5] [6]. The medicinal plants used in TCM have attracted the western academic research community in the last decades as they are source of various drugs for several clinical disorders [7]-[12]. Regarding global medicinal plants on the planet, there are a very small number of plants that have been analyzed for their biochemical composition and therefore, it is crucial to carry out further research on new medicinal plants to search for novel biological properties.

The olive tree (*Olea europaea*) belongs to the *Oleaceae* family [3], with elongated leaves, which is found in Mediterranean region for over 7000 years ago [13]. The olive production has been increasing in the last 3 decades (from around 1500 million tons in 1990 to 3300 million tons in 2018); a similar trend was recorded for the consumption of olive oil worldwide, which reached 3000 million tones [14]. Throughout the history of mankind, *Olea europaea* leaves have been widely exploited for prevention or treatment of hypertension, carcinogenesis, diabetes, atherosclerosis and several other traditional therapeutic uses [3] [15]-[20]. The overall incidence of cancer in the Mediterranean basin has been considered to be the lowest compared to the other parts of the planet namely for breast, endometrium, prostate, colon and leukemia cancer [3] [15]. Historically, olive leaf has also been used as a remedy for fever and other diseases such as malaria [21].

In the harvest process of olives, leaves are also part of the harvested product (*i.e.* represent around 10% of the total weight product which arrives to the mill), being later separated as a co-product with no value and use. Thus, any study that could create an added-value for the leaves would make the olive oil value-chain more competitive.

The main phenols occurring in *O. europaea* are secoiridoids which are complex substances with a molecular weight up to 600. These compounds contain one or two hydroxy-aromatic rings which are bound to several other non-aromatic compounds [22].

Olive leaf contains several major active biomolecules which are oleuropein and its derivatives such as hydroxytyrosol and tyrosol, as well as vanillin, luteolin,

diosmetin, rutin, luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, caffeic acid, p-coumaric acid and vanillic acid [23]. The concentration of phenols was found to be several times higher in leaves compared to various oil fractions, and its content varied with time and climate of olive trees [23].

Although there are several reports in the literature about the anti-cancer activity of olive leaf extracts (OLE) [23], there is no research work in the literature about the: 1) Effect of OLE from Portuguese olive cultivars in four human carcinoma cell lines: HeLa, A549, A431 and EO21; 2) Comparative analysis of the anti-cancer activity of young (Y) and old (O) olive leaves; 3) Effect of freeze-dried olive leaves on human carcinoma cell lines.

The aim of the present work consists of extraction of phenolic compounds from freeze-dried and fresh green olive leaves from five different Portuguese varieties of olive trees of *Olea europaea*—Madural, Cobrançosa, Arbequina, Verdial and Galega. Y and O olive leaves were used to prepare leaf extractions. These extracts were used at different concentrations in human carcinoma cell lines in order to study cell inhibition growth by using tetrazolium dye (MTT) colorimetric assay.

2. Experimental Procedures

2.1. Reagents, Cell Lines and Olive Leaves

Fetal bovine serum (FBS), gentamycin, penicillin, streptomycin, DMSO, gallic acid, RPMI, Folin-Ciocalteu, Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma Chemical Company (S. Louis, MO, USA).

Human carcinoma cell lines HeLa (Cervix), A549 (Lung), A431 (Epidermis) and OE21 (Oesophagus) were obtained from European Collection of Cell Cultures (ECACC).

Olive leaves were collected from trees with 20-year old, in the Experimentation Research Farm of the Ministry of Agriculture in Mirandela, Portugal (41.4854˚N, 7.1809˚W), the core region for the olive production, where a collection of cultivars is preserved following a comprehensive identification by botanists and agronomists from the Ministry of Agriculture, Portugal. This field is preserved to monitor the performance of each cultivar and to supply vegetative material to local nurseries. The five varieties were Madural, Cobrançosa, Arbequina, Verdeal and Galega from Mirandela, Portugal, containing both young (Y) and old (O) leaves. Young leaves were formed during the last year before harvest whereas the oldest leaves were 3-year old in the branch/tree.

All other reagents used were of analytical grade.

2.2. Extraction of Phenolic Compounds

The extraction of phenolic compounds was adapted from Reference [23]. Freeze-dried and fresh green olive leaves, provided by the department of Agronomy,

UTAD were used and two types of leaves were extracted (*i.e.* O and Y leaves) in order to find out how their phenolic content would be affected. All leaves were washed with distilled water, weighed before and after the freeze-drying procedure.

10 g of either fresh or freeze-dried leaves were ground to a size of 0.1 mm and extracted with 70% ethanol (100 mL) for 2 weeks in the dark in an orbital shaker at 100 rpm at room temperature. The suspension [10% (w/v)] was filtered with a Millipore filter (pore size 0.22 μm) and stored in the dark at 4˚C.

2.3. Assay of Phenolic Compounds

Phenolic compounds in OLE were assayed according to [24], with some major modifications. Suitable diluted OLE (100 μL), different concentrations of gallic acid as standard (0 - 100 μg/mL), and blank solutions were pipetted into eppendorf tubes and Folin-Ciocalteau reagent (100 μL) was added to each tube. The mixture was mixed by vortex and after 2 min, 5% (w/v) sodium carbonate solution (800 μL) was added to eppendorf tubes and the mixture was vortexed and incubated in a water bath at 40˚C for 20 min. The eppendorf tubes were cooled on ice bath and the samples were read at A765 which was read in a glass cuvette in a Thermo Evolution 300 LC UV/visible spectrophotometer. All assays were carried out in triplicate.

2.4. Growth and Maintenance of Human Carcinoma Cell Lines

Cell lines were grown in tissue culture flasks and were maintained in the appropriate culture medium in a CO_2 incubator at 37˚C containing 5% CO_2.

As far as cell lines A432, A549 and HeLa were concerned, cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) containing gentamycin and 10% fetal bovine serum (FBS) whereas the cell line OE21 was grown in RPMI (Roswell Park Memorial Institute medium) medium, supplemented by FBS, penicillin and streptomycin. Cell detachment from the flasks was carried out by adding 0.01% (w/v) trypsin to release cells from the flask which was gently mixed and incubated for about 3 minutes at 37˚C. A suitable amount of previously warmed culture medium at 37˚C was added to the flask and the cell suspension was transferred to a tube which was centrifuged for 6 minutes at × 448 g. The supernatant was removed and fresh culture medium was added to the pellet which was resuspended and viable cell concentration was determined in these samples. Cell suspensions were treated with 0.1% (w/v) trypan blue in PBS and viable cell concentration was determined by the Neubauer improved method.

2.5. MTT Standard Curves for Human Carcinoma Cell Lines

Viable cell numbers were quantified in either 24 or 96-well culture plates by using the MTT colorimetric assay. Standard curves were prepared by plating various concentrations of isolated cells which were previously quantified by Newbauer improved hemocytometer [25], in triplicate assays in microtiter 96-well

tissue culture plates [26].

2.6. Effect of Phenolic Compounds on Proliferation of Human Carcinoma Cell Lines (*in Vitro* Anti-Tumor Activity Assay)

Colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used for measuring the proliferation of human carcinoma HeLa, A549, A431 and OE21 cell lines in microtiter plates of 96 wells containing the appropriate culture medium supplemented with 10% fetal bovine serum and gentamycin [27]. Preliminary experiments were also carried out with normal human lung cell line.

Phenolic compounds from freeze-dried leaves were dissolved in either DMEM or PBS at different concentrations in the range of 0.07 - 2.4 µg phenolic compounds/well (*i.e.* 7 - 240 µg/mL) and they were filtered by a Millipore 0.22 µm filter before addition to the cells in order to maintain sterility. On the other hand, phenolic compounds from fresh leaves were used with no dilution at a single concentration in the range of 2.09 - 8.44 µg phenolic compounds/well (*i.e.* 209 - 844 µg/mL).

Single concentrations and several concentrations of phenolic compounds from fresh leaves and freeze-dried leaves (10 µL) respectively, were added to each well containing 5.5×10^4 cells per well and the cultures were incubated in a CO_2 incubator with 5% CO_2 at 37˚C for 48 h. Cellular viability was determined by the addition of 20 µL MTT (5 mg/mL) to each microtiter wells and after 4 h incubation, the supernatant was removed and 200 µL DMSO was added to each well to solubilize the precipitate. The rate of viable human carcinoma cell line was determined by measuring A550 in a microtiter plate reader. These results were expressed as the inhibition ratio (I) of human carcinoma cell line proliferation as follows: $I = [X - Y]/X * 100\%$.

X and Y are the average number of viable cancer cells of the control (*i.e.* either culture medium or PBS) and test samples, respectively. All assays were carried out in triplicate.

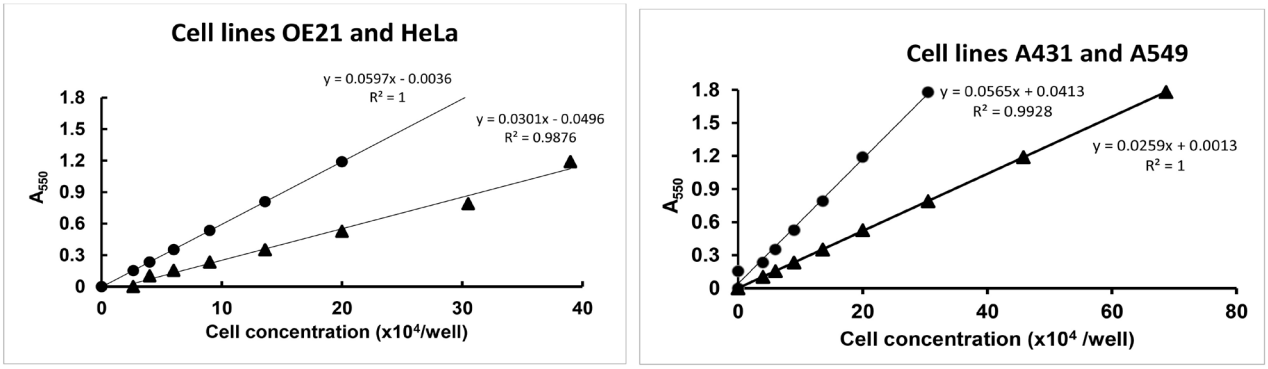
2.7. Statistical Analysis

SigmaPlot 12.0 (2011-2012 Systat Software inc.) was used to draw graphs in the present work. Experimental results are means of three parallel measurements and the results are presented as mean values ± standard deviation (SD). Correlation and regression analyses were performed with the Excel software 2013 package (Academic License, Microsoft of Portugal). Correlations were considered statistically significant at $p < 0.05$ according to Tukey HSD and Scheffé test.

3. Results

3.1. MTT Standard Curves for Human Carcinoma Cell Lines

The data presented in **Figure 1(a)** and **Figure 1(b)** revealed the standard curves



(a) (b)

Figure 1. MTT standard curve for four human carcinoma cell lines used in this research work and values represented are mean of triplicates. (a) OE21 (●) and HeLa (▲); and (b) A431 (●) and A549 (▲).

for all four human carcinoma cell lines. There is a linear relationship between A550 and cell concentration for each cell line (**Figure 1**).

3.2. Phenolic Compounds

Fresh and freeze-dried leaves were used to extract phenolic compounds which exhibited levels in the range of 2.09 ± 0.10 to 8.44 ± 0.64 and 7.72 ± 0.56 to 24.65 ± 1.9 mg gallic acid equivalents/g leaves, respectively. The data presented in **Figure 2** strongly suggests that freeze-dried leaves exhibited higher phenolic contents than fresh leaves. This result may be explained due to the fact that this treatment removed most of water in leaves at low temperature. Moreover, freeze-drying procedure has been reported to exhibit high concentration of phenolic compounds in spearmint compared with other drying methods which may be due to loss of heat-sensitive phenolic compounds [28]. Regarding the differences between Y and O leaves, it was found that the Y Cobrançosa sample exhibited 50% higher phenolic content compared with the O leaves (**Figure 2**). Moreover, the levels of phenolic compounds in freeze-dried leaves were also higher in Y Cobrançosa compared with the O leaves. Another interesting result was observed for O Verdeal leaf extract which exhibited an increase in phenolic contents compared with the Y leaf extract both for fresh and freeze-dried leaves. The phenolic content of the O and Y leaves of the other olive tree species (*i.e.* Madural, Galega and Arbequina) did not differ significantly (**Figure 2**).

3.3. Effect of Phenolic Compounds on Proliferation of Human Carcinoma Cell Lines

Preliminary experiments were carried out with normal human lung cell line and OLE did not affect the proliferation of such cell line (data not shown). The effect of addition of either PBS or DMEM to cell wells *in vitro* was investigated which revealed no cell toxicity (data not shown).

The data reported in **Figures 3-6** have revealed that the effect of phenolic compounds on growth inhibition of human carcinoma cell lines was dependent on the olive tree cultivar, cell line and age of olive leaves.

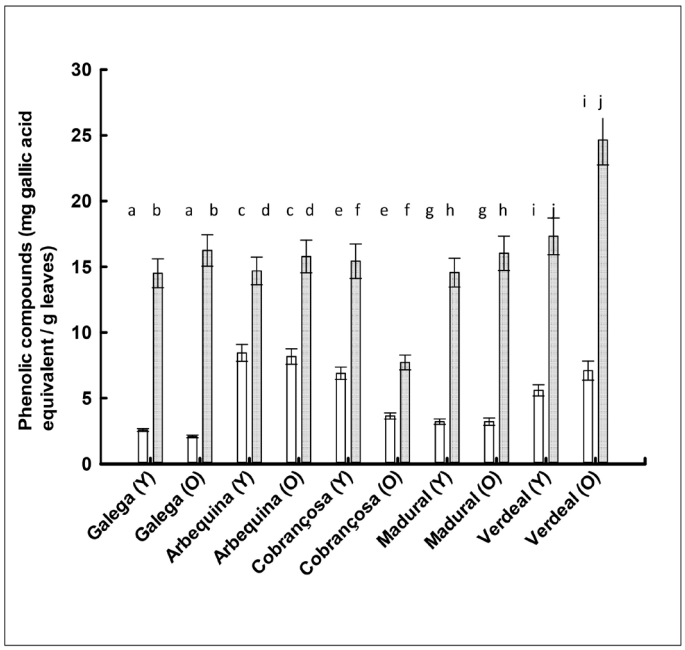


Figure 2. Assay of phenolic compounds in fresh leaves (open columns) and freeze-dried (blue columns) leaves. The data are presented as mg of gallic acid equivalents/g of either fresh or freeze-dried leaves. Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

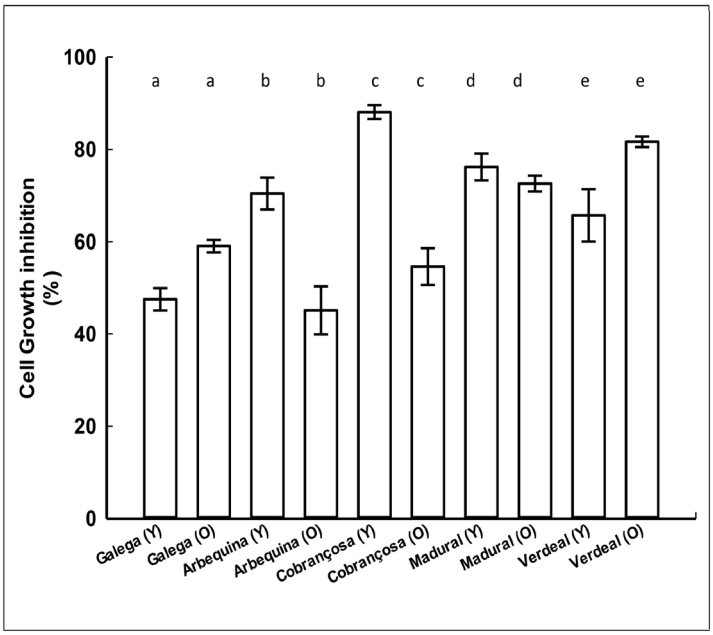


Figure 3. Cell growth inhibition (%) by addition of olive phenolic compounds with no dilution at a single concentration in the range of 2.09 - 8.44 μg phenolic compounds/well (*i.e.* 209 - 844 μg/mL) from fresh leaves to HeLa human carcinoma cell line; mean of triplicate values and error bars are represented. Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

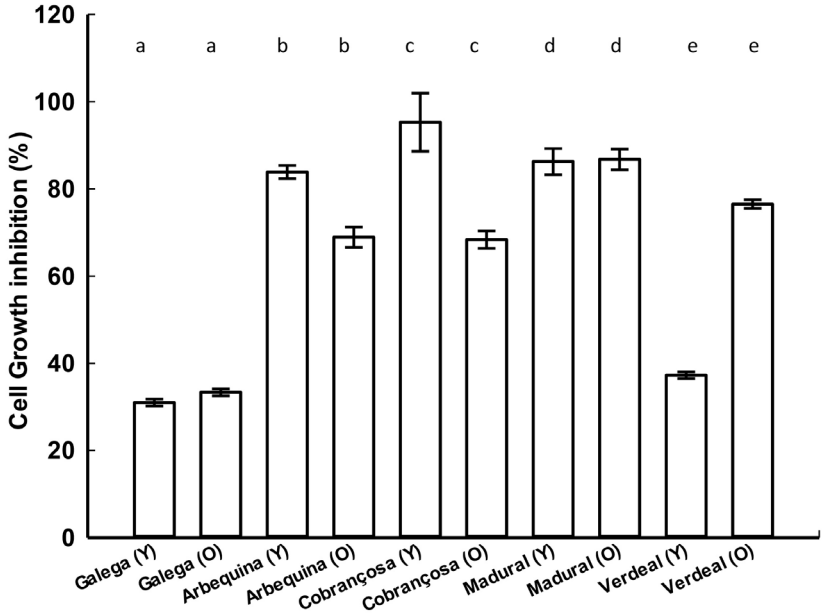


Figure 4. Cell growth inhibition (%) by addition of olive phenolic compounds with no dilution at a single concentration in the range of 2.09 - 8.44 μg phenolic compounds/well (*i.e.* 209 - 844 μg/mL) from fresh leaves to A549 human carcinoma cell line; mean of triplicate values and error bars are represented; Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

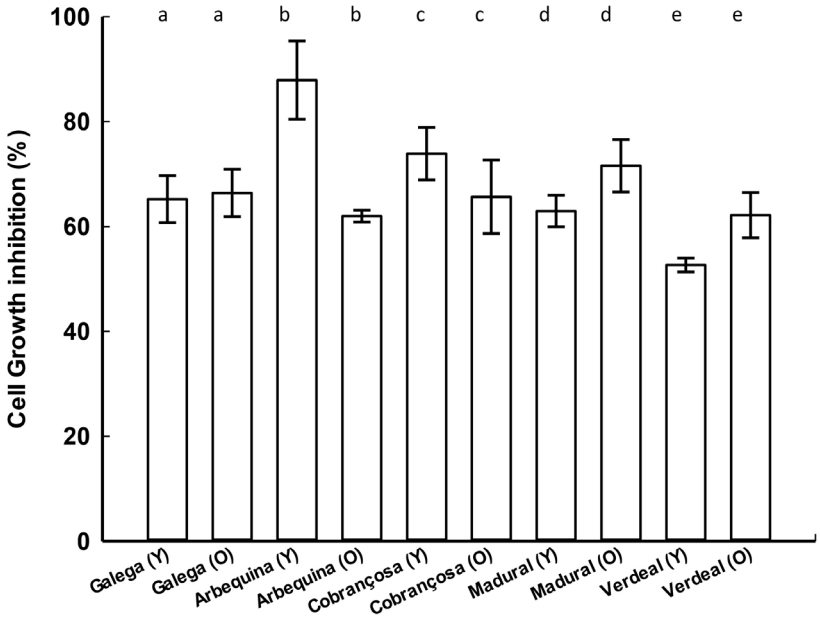


Figure 5. Cell growth inhibition (%) by addition of olive phenolic compounds with no dilution at a single concentration in the range of 2.09 - 8.44 μg phenolic compounds/well (*i.e.* 209 - 844 μg/mL) from fresh leaves to A431 human carcinoma cell line; mean of triplicate values and error bars are represented; Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

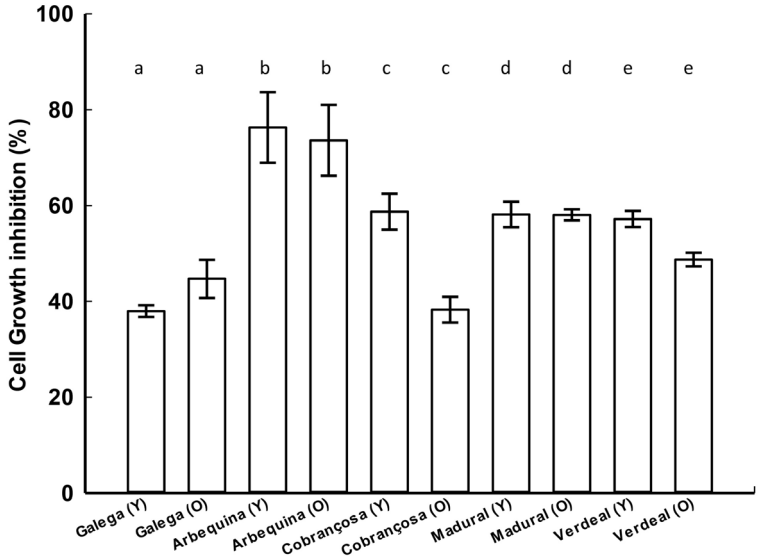


Figure 6. Cell growth inhibition (%) by addition of olive phenolic compounds with no dilution at a single concentration in the range of 2.09 - 8.44 μg phenolic compounds/well (*i.e.* 209 - 844 μg/mL) from fresh leaves to OE21 human carcinoma cell line; mean of triplicate values and error bars are represented. Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

In general, Y leaves have revealed the highest cell growth inhibition ranging from about 95% for Cobrançosa (Y), followed by 90% for Cobrançosa (Y), 90% for Arbequina (Y) and 75% for Arbequina (Y) for cell lines A549, HeLa, A431 and OE21, respectively (Figures 3-6). Moreover, Cobrançosa (Y) leaf extract exhibited the highest cell growth inhibition followed by Arbequina (Y) leaf extract. The lowest cell growth inhibition was observed for Galega (Y) leaf extract (Figures 3-6). Therefore, the data presented in this work strongly suggest that there are marked differences in both types of leaf extracts which may be due to the presence of different phenolic compounds in such leaves (Figures 3-6).

The effects of phenolic compounds from freeze-dried leaves on the proliferation of two cell lines are shown in Figure 7 and Figure 8 which present a rather interesting pattern as far as the degree of dilution of leaf extracts are concerned. As mentioned above (Figure 2), the amount of phenolic compounds in freeze-dried samples was found to be two to three-fold higher than in fresh olive leaf extract. Freeze-dried leaf extracts were applied to human carcinoma cell lines A431 and A549 and the result, in general, exhibited a distinctive but rather persistent pattern since cell growth inhibition was highest at highest extract dilution (Figure 7 and Figure 8).

In fact, neat extracts from freeze-dried leaves did not reveal a significant cell growth inhibition for these cell lines (data not shown). However, substantial inhibitory effect on cell proliferation was only found to occur when the leaf extracts were diluted at least 10-fold as shown in Figure 7 and Figure 8. As far as A431 cell line is concerned, the highest cell growth inhibition was 75% for Galega (O),

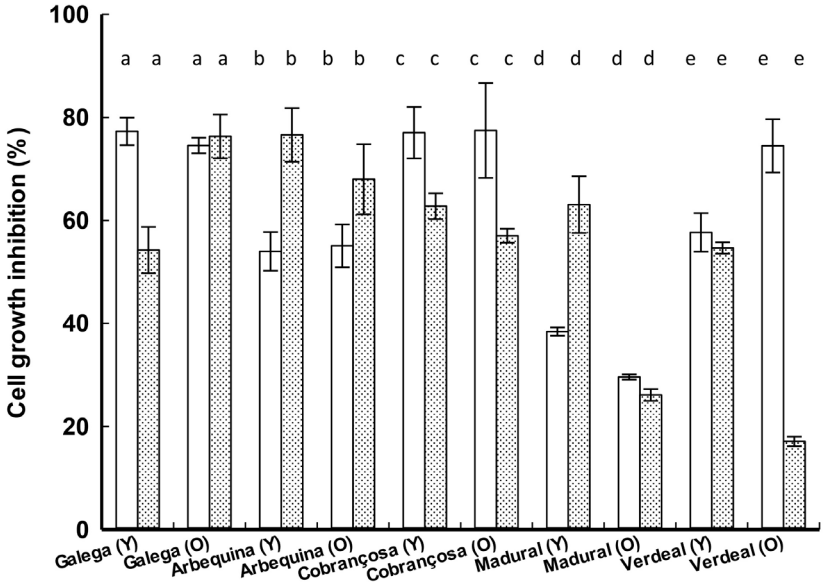


Figure 7. Addition of freeze-dried olive phenolic compounds at different concentrations in the range of 0.14 - 4.8 μg phenolic compounds/well (*i.e.* 14 - 480 μg/mL) to A431 human carcinoma cell line. The dotted bars represent a 1:10 dilution in PBS/DMEM and non-dotted bars represent 1:50 dilution in PBS/DMEM; mean values and error bars are represented. Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

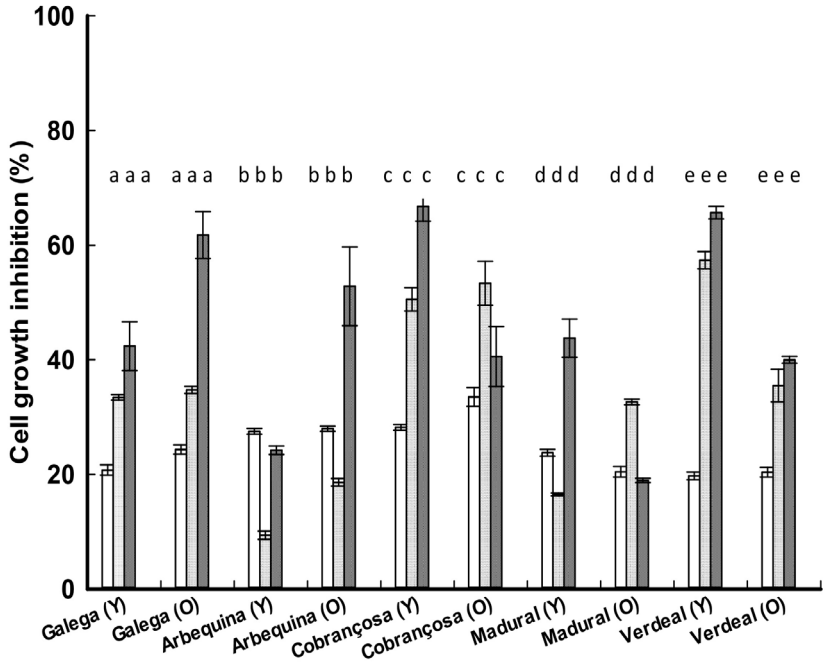


Figure 8. Addition of freeze-dried olive phenolic compounds at different concentrations in the range of 0.07 - 2.4 μg phenolic compounds/well (*i.e.* 7 - 240 μg/mL to A549 cell line. The dark gray bars represent a 1:100 dilution in PBS/DMEM, light blue a dilution of 1:50 in PBS/DMEM and non-dotted bars a 1:10 dilution in PBS/DMEM; mean values and error bars are represented. Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

followed by Arbequina (Y), Cobrançosa (Y), Madural (Y) and Verdeal (Y) (**Figure 7**). Regarding A549 cell line, the highest cell growth inhibition was 70% for Cobrançosa (Y), followed by Verdeal (Y), Galega (O), Arbequina (O) and Madural (Y) (**Figure 8**).

4. Discussion

MTT standard curves for four human carcinoma cell lines were carried out *in vitro* and there is a linear relationship between A550 and cell concentration for each cell line. It was found that the behavior of standard curve was slightly different from cell line to cell line as shown in their equations. Similar results have been obtained from other research workers by using different human carcinoma cell lines [26] [29].

Fresh and freeze-dried leaves were used to extract phenolic compounds and the data presented in **Figure 2** strongly suggests that freeze-dried leaves exhibited higher phenolic contents than fresh leaves. Similar results have been reported in the literature from *Coratina* olive cultivar leaves which exhibited 67 ± 2.0 and 108 ± 2.0 mg equivalents gallic acid/g leaves or fresh and freeze-dried leaves, respectively [30]. The data published in the literature is difficult to compare with the present work since different extraction procedures were used such as high temperature, different solvents and extraction techniques [19] [22] as well as different olive leaf cultivars. Except for Cobrançosa and Verdeal cultivars, the levels of phenolic compounds were similar between Y and O leaves, which is in agreement with other findings [31]. In fact, it has been found that phenolic contents in different aged olive (*Olea europaea L.*) cultivar leaves exhibited similar levels [31]. The levels of phenolic compounds in fresh leaves obtained in the present work were slightly lower than the data reported in the literature for Portuguese olive leaf cultivars (*i.e.* 11.6 - 17.4 and 11.7 - 40.1 g tannic acid equivalents/kg for fresh and dried leaves (40˚C), respectively [32]. However, this difference may be due to several reasons since different assay conditions, different extraction procedure of phenolic compounds and different olive leaf cultivars were used in their report [32] compared with the present work.

The effect of phenolic compounds from fresh leaves on proliferation of human carcinoma cell lines *in vitro* exhibited significant cell growth inhibition. It is important to stress that these phenolic compounds were used at a single concentration with no dilution on human cell lines. As far as freeze-dried extracts are concerned, it was consistently found that neat extracts with no dilution of freeze-dried leaves exhibited no growth inhibition of human carcinoma cell lines (data not shown). However, dilutions of these extracts revealed growth inhibition of cell lines A431 and A549 which was highest at the highest dilution factor. Moreover, the effect of extract dilution was more pronounced on growth inhibition when initial lower cell concentrations (1×10^4 cells/well) were used (data not shown). In other words, there is a non-linear growth inhibition of human cell lines as a function of phenolic compound concentration from freeze-dried

extract. As far as literature is concerned, there is a report on such non-linear behavior by using Siberian ginseng (*Eleutherococcus senticosus*) extracts on human carcinoma cell lines [33]. To authors knowledge, there are no reports in the literature about the: 1) Effect of OLE from Portuguese olive cultivars in four human carcinoma cell lines: HeLa, A549, A431 and EO21; 2) Comparative analysis of the anti-cancer activity of young (Y) and old (O) olive leaves; 3) Effect of freeze-dried olive leaves on human carcinoma cell lines. However, there are several published works about the inhibitory effect of OLEs on growth of human carcinoma cell lines [19] [23] [34] which are in general agreement with the data presented in this work. But it is difficult to compare published data with the present work since different human cell lines, different extraction procedures, different olive leaf ages and different olive leaf cultivars were used.

5. Conclusions

OLE were obtained from different olive leaf cultivars as well as from different leaf growing stages. The assay for phenolic compounds in these extracts revealed higher levels in freeze-dried leaves compared with the fresh leaves.

These OLE from several Portuguese olive cultivars were used to study their effect on growth of human carcinoma cell lines *in vitro*. The data presented in this work strongly suggest that they inhibited the growth of human carcinoma cell lines. In general, Y leaves have revealed the highest cell growth inhibition ranging from about 95% for Cobrançosa (Y), followed by 90% for Cobrançosa (Y), 90% for Arbequina (Y) and 75% for Arbequina (Y) for cell lines A549, HeLa, A431 and OE21, respectively. Moreover, Cobrançosa (Y) leaf extract exhibited the highest cell growth inhibition followed by Arbequina (Y) leaf extract. However, freeze-dried samples exhibited a distinctive pattern since cell growth inhibition was highest at highest extract dilution.

Further work is required to carry out chromatographic fractionation of the crude leaf extract in order to obtain individual column fractions which must be analyzed on the proliferation of human carcinoma cell lines *in vitro*. These secondary metabolites responsible for growth inhibition must be identified by analytical techniques such as FTIR, NMR, HPLC and GC-MS. And finally, the molecular mechanism responsible for growth inhibition of human carcinoma cell lines must be investigated in detail by using molecular biology and immunochemical techniques.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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