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# Evaluation of Cytotoxic and Antioxidant Activities of Purified Vegetal Extracts and Its Main Pure Compounds

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**Abstract**

Vegetal aqueous extracts are an important source of phytochemicals with biological activities. Concretely, olive leaf extract (OLE) and green tea extract (GTE) contain a high amount of phenolic compounds that present antioxidant, antibacterial and cytotoxic properties, among others. As described in literature, plant extracts containing photosynthetic pigments, such as chlorophylls, may cause an undesirable pro-oxidant effect when applied in food matrices. Such pro-oxidant effect, however, must be analysed according to the final applicability of the extract.

The aim of this work was to purify natural extracts' phenolic content by elimination of chlorophyll compounds and evaluate the cytotoxic and antioxidant activity of the extracts, the importance of the individual major phenolic compounds and the influence of chlorophylls. The extracts were characterized by HPLC which showed that they contained different phenolic compounds in high concentration, particularly, OLE contained mainly hydroxytyrosol, oleuropein and tyrosol, while GTE contained caffeine, gallocatechin, epigallocatechin, epigallocatechin gallate, gallocatechin gallate as main phenolics. Afterwards, extracts were subjected to purification process by column chromatography and the amount of phenolic compounds decreased because the column yield was 33-58%.

The antioxidant capacity of the extracts was evaluated by ABTS method. Pro-oxidant or anti-oxidant activity of individual phenolic compounds were concentration-dependent because when the extracts were doped with individual compounds, this fact generated a decrease in antioxidant activity compared to fresh extracts. The individual phenolic compounds did not show synergic effect when combined, and the elimination of chlorophyll compounds did not generate a change in the antioxidant activity of extracts, suggesting that these vegetal pigments are not relevant to such capacity. On the other hand, the cytotoxic activity of the extracts was tested against HL-60 leukemic cells using the MTS tetrazolium salt reduction assay. The results showed that both OLE and GTE had a high antiproliferative activity, 66.3% and 70.1%, respectively and, in contrast to their antioxidant activity, the elimination of chlorophyll compounds generated an important decrease of cytotoxic effect in both cases. Only hydroxityrosol and caffeine phenolic compounds individually showed important antiproliferative activity, 44.5% and 25.2%, respectively. But when the fresh extracts were doped with some individual compounds, the cytotoxic capacity was increased. This can be explained by the extract's matrix effect on the individual compounds.

In conclusion, these results highlight the great antioxidant and antiproliferative potential of the natural extracts tested. In addition, these findings showed that photosynthetic pigments acquired a considerable relevance in the cytotoxic capacity of OLE and GTE.

**Keywords:**

Green Tea;

Olive Leaf;

Pro-oxidant;

Chlorophyll;

Bioactivity.

## 1. Introduction

Over the years, plants have been an important source of bioactive and phytochemical compounds which have been used for application in many fields, including medicine and food industry. A large number of functional and nutraceutical ingredients are currently being developed from plant derivatives and their phytochemicals. Medicinal plants used as food for the prevention and/or control of diseases are natural sources of antioxidant substances such as phenolic compounds [1]. Epidemiological studies have shown that people who eat diets rich in fruits and vegetables have a lower risk of degenerative diseases, such as cancer, cardiovascular disease and diseases derived from these [2], among many others. These potential benefits for humans are attributed to their phytochemical components such as vitamins, lycopenes, carotenoids and phenolic compounds.

Phenolic compounds are secondary metabolites with a considerable physiological and morphological importance in plants. Our body cannot naturally synthesize phenolic compounds, so they must be incorporated into the diet along with

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other essential nutrients in order to enjoy their benefits. This group of compounds mainly possesses antioxidant activity to prevent the oxidation damage by reactive species [1], avoid the initiation stages of cancer [3], and exert positive effects against cardiovascular problems [4]. Besides, some authors have described pro-oxidant effects of this molecules [5], a capacity that needs to be studied in depth to determine its mechanisms and the possible future applicability due to its relationship with anti-cancer properties.

These bioactive molecules have been found in important concentrations in many parts of plants such as olive leaves [6] and green tea leaves [7]. The major individual phenolic compounds identified in olive leaves aqueous extract (OLE) are caffeic acid, hydroxytyrosol, quercetin, luteolin, ligstroside, oleuropein, demethyloleuropein, vanillic acid, verbascoside and tyrosol [6]. The main responsible for its antioxidant properties is oleuropein [8], a secoiridoid recognized by European Food Safety Authority (EFSA) as suitable substance for use in food supplements due to its health benefits, such as increase in glucose tolerance. Moreover, it has been described that oleuropein and hydroxytyrosol (phenolic alcohol) decrease the number of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis [9]. Other biological properties of OLE include its antiviral [10] and antimicrobial activity against human pathogens [11].

On the other hand, Rusak et al. [7] confirmed that green tea is a richer source of phenolics than other tea species, in which eight catechins (epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin, gallocatechin gallate, gallocatechin, catechin gallate and catechin), caffeine and eight other minor phenolic compounds are identified [12]. These major phenolic compounds in green tea mainly present antioxidant and anti-cancer properties. Catechins, when combined with anti-cancer compounds, have anti-cancer capacity against numerous cancer cell lines as human head, neck, lung, breast, prostate, liver, ovarian, leukemia, malignant neuroblastoma and various organs cancer cells [13]. Its mechanisms of action are very varied including the induction of apoptosis by enhancing the expression of genes that regulate it, synergistic inhibition of cell proliferation and colony formation, and inhibition of kinases as well as activation of caspases, among many others. Three of the main green tea catechins, namely EGCG, EGC and ECG, have cancer-preventive activity, and the combination of them has synergistic effects on the induction of apoptosis and inhibition of cell growth in PC-9 cells, and inhibits the release of TNF-α, an endogenous tumor promoter [14]. Concretely, it has been described the synergistic enhancement of apoptosis, gene expression, and anti-cancer effects using various combinations of EGCG and anti-cancer drugs such as COX‐2 inhibitors [15]. Also, catechins possess other properties such as antimicrobial and anti-inflammatory actions, among many others [16].

Furthermore, plant extracts contain photosynthetic pigments such as chlorophylls, which are necessary for the absorption of sunlight in photosynthesis. Chlorophyll and its various derivatives present biological activities such as cancer prevention, induction of apoptotic events, antioxidant activity and antimutagenic activity. These encouraging results have led to the investigation of their chemopreventive effects in humans [17]. Due to their light-absorbing properties, the energy absorbed by these molecules can be transferred to oxygen, resulting in the production of reactive oxygen species (ROS) and thus presenting pro-oxidant activity. In addition, Wanasundara and Shahidi [18] reported that the presence of chlorophyll in green tea extracts (GTE) was responsible for their pro-oxidant effect on the oxidation of marine oils. Nonetheless, not only the chlorophylls are responsible for such pro-oxidant activity, the concentration of individual phenolic compounds can also generate this effect [5].

The pro-oxidant properties of chlorophylls and catechins [19] are relevant for the anticancer capacity of these extracts. This is a field worth to be investigated to clarify its mechanisms of action for a putative application in cancer therapy. Many studies have evaluated the properties of bioactive compounds individually, but these compounds are not isolated in nature but interact with each other, thereby enhancing their activity in some cases. This is the so-called synergic effect. On the other hand, these compounds can act in one way or another depending on the medium in which they are located and the composition thereof. The modification of its properties depending on the set of molecules that surround them is known as matrix effect.

Therefore, the aim of the present study was to investigate the cytotoxic and antioxidant capabilities of an OLE and a GTE before and after a purification process to eliminate their chlorophylls. Additionally, it was intended to determine the possible synergic or matrix effects of the main phenolic compounds of the extracts and the influence of the chlorophylls on these bioactivities.

## 2. Materials and methods

### *2.1. Preparation of raw material*

Olive leaves were picked up from a local company (Badajoz, Spain). The samples were immediately transported to the laboratory in ventilated storage trays, vacuum-packaged (Gustav Müller VS 100, Germany) in plastic bags (500 gr) and frozen until their use (-80ºC). These processes were carried out with care to avoid changes in the composition of the material. Dried green tea leaves were obtained from a local market.

### *2.2. Extraction of bioactive compounds from extracts*

Before extraction treatment, fresh olive leaves were washed with distilled water and partially dried (model 210, Selecta® P, Spain) during 12 min at 120ºC. Dried samples (green tea and olive leaves) were ground in a domestic knife mill to obtain particles (0.5-3.0 mm). Then, bioactive compounds were extracted with water (1:10 w/v) at 118ºC for 15 min, the samples were filtered and centrifuged to remove solid particles. Finally, the extracts were frozen at -80°C until

further analysis. The extracts purification was carried out using a column (1.25 cm internal diameter and 20cm height) packed with Toyopearl HW-40F (Tosoh Bioscience LLC) was used. Crude extracts were dissolved in 80% (v/v) aqueous ethanol and introduced onto the column. The column was eluted with hexane until all the residual green colour had disappeared. The column was then washed with 80% (v/v) aqueous ethanol to recover the dechlorophyllized green tea extracts. Ethanol was removed by evaporation under vacuum at 40°C using a rotary evaporator and the residual water was removed by lyophilization.

### *2.3. Experimental design*

The extracts were analysed in crude and after the purification process. The composition of phenolic compounds, the antioxidant and cytotoxic activities were evaluated to detect changes in the bioactive compounds present in the extracts. In order to evaluate the possible synergic or matrix effect on the antioxidant and cytotoxic activities of individual phenolic compounds present in GTE and OLE, the extracts were diluted (1:10 v/v) and mixed with standards of oleuropein, hydroxytirosol (Extrasynthése, Genay, France) and tyrosol (Sigma-Aldrich Chemie, Steinheim, Germany) in the case of OLE, and with caffeine (Enzo Biochem, Farmingdale, USA), gallocatechin (Extrasynthése, Genay, France), epigallocatechin, gallocatechin gallate (Chengdu Biopurify Phytochemicals, Sichuan, China) and epigallocatechin gallate (Adipogen, Liestal, Switzerland) in the case of GTE. The combinations analysed are summarized in table 1 and table 2.

**Table 1. Experimental design followed to evaluate the matrix effect between phenolic compounds of GTE. Individual standards of phenolic compounds, a mixture of them (multi-standard) as well as diluted GTE mixed with standards were evaluated.**

| | Caffeine (mg/kg) | Gallocatechin (mg/kg) | Epigallocatechin (mg/kg) | Epigallocatechin gallate (mg/kg) | Gallocatechin gallate (mg/kg) | Abbreviations |
|---|---|---|---|---|---|---|
| **Multi-standard** | 2000 | 2000 | 2000 | 2000 | 2000 | TMIX |
| **Individual Standards** | 2000 | - | - | - | - | CA |
| | - | 2000 | - | - | - | GC |
| | - | - | 2000 | - | - | EGC |
| | - | - | - | 2000 | - | EGCG |
| | - | - | - | - | 2000 | GCG |
| **Fresh extract** | - | - | - | - | - | GTEC |
| | 2000 | - | - | - | - | GTEC+CA |
| | - | 2000 | - | - | - | GTEC+GC |
| | - | - | 2000 | - | - | GTEC+EGC |
| | - | - | - | 2000 | - | GTEC+EGCG |
| | - | - | - | - | 2000 | GTEC+GCG |
| **Purified extract** | - | - | - | - | - | GTENC |
| | 2000 | - | - | - | - | GTENC+CA |
| | - | 2000 | - | - | - | GTENC+GC |
| | - | - | 2000 | - | - | GTENC+EGC |
| | - | - | - | 2000 | - | GTENC+EGCG |
| | - | - | - | - | 2000 | GTENC+GCG |

**Table 2. Experimental design followed to evaluate the matrix effect between phenolic compounds of OLE. Individual standards of phenolic compounds, a mixture of them (multi-standard) as well as diluted OLE mixed with standards were evaluated.**

| | Oleuropein (mg/kg) | Tyrosol (mg/kg) | Hydroxytyrosol (mg/kg) | Abbreviations |
|---|---|---|---|---|
| **Multi-standard** | 2000 | 2000 | 2000 | OMIX |
| **Individual Standards** | 2000 | - | - | O |
| | - | 2000 | - | TY |
| | - | - | 2000 | HY |
| **Fresh extract** | - | - | - | OLEC |
| | 2000 | - | - | OLEC+O |
| | - | 2000 | - | OLEC+TY |
| | - | - | 2000 | OLEC+HY |
| **Purified extract** | - | - | - | OLENC |
| | 2000 | - | - | OLENC+O |
| | - | 2000 | - | OLENC+TY |
| | - | - | 2000 | OLENC+HY |

### *2.4. HPLC analysis of phenolic compounds of the OLE and GTE before and after purification*

Standards of polyphenolic compounds were prepared in methanol and stored in darkness at -20ºC. HPLC mobile phases were prepared with HPLC grade methanol and acetonitrile (Fisher Chemical, Loughborough, UK) and P.A. grade formic acid (PANREAC, Barcelona, Spain). The HPLC analysis was carried out following the method and conditions described by Cabrera-Bañegil et al. [20]. Main phenolic compounds analysis was carried out with an Agilent 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany), which was equipped with a diode array detector (DAD) and fluorescence detector (FLD). Also, for this determination, a Gemini-NX C18 column was used (150x4.6 mm i.d., 3 µm thickness, Phenomenex).

### *2.5. Antioxidant activity assays of the extracts*

The antioxidant capacity of the extract samples was determined by the ABTS•+ method [21]. In this assay, 100 µl of ABTS solution (2.2'-azinobis (3-ethylbenzoithiazolone 6-sulphonate)) were mixed with different combinations of extracts samples (Tables 1 and 2) and these mixtures were added to 96-well microtiter plates. Absorbance was measured at 730 nm and results were expressed as mmol Trolox 10 $mL^{-1}$ using a calibration curve of Trolox.

### *2.6. Cell culture*

Human promyelocytic leukemia HL-60 cell line (ECACC No. 88120805; Dorset, UK) was grown in RPMI 1640 medium (Lonza) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µ/mL streptomycin. Cells were cultured in a humidified atmosphere containing 5% $CO_2$ at 37ºC.

### *2.7. In vitro cytotoxicity assay*

The cytotoxic effects of the different extracts were assayed on HL-60 cell line by means of the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madrid, Spain), which is based on the reduction of an MTS tetrazolium compound. Cells were seeded in 96-well plates at a density of $2\times10^4$ cells/well. After treating cultures for 24h, assays were performed by adding 10 µl of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 2h at 37 ºC, and then recording absorbance on a microplate reader (Infinite M200; Tecan Austria GmbH, Groedig, Austria) at a test wavelength of 490 nm and a reference wavelength of 650 nm to subtract background. All analyses were run in triplicate. The cell viability was calculated as percentage of control values (untreated samples).

### *2.8. Statistical analysis*

Three replicates were carried out for each treatment and analysis. The results were represented as means and standard deviations and were analysed by SPSS 18.0 statistical software (SPSS Inc., Chicago, IL). One-way analysis of variance

(ANOVA) was used to compare the data, and all the tests were considered statistically significant at $p<0.05$. When analysis detected significant differences, means were compared using Tukey's test and Student's t-test.

## 3. Results and discussion

### *3.1. Phenolic compounds content of extracts and changes after purification*

The quantification of the main phenolic compounds content in OLE and GTE before and after purification are shown in Table 3.

**Table 3. Concentration of main phenolic compounds of OLE and GTE (mg/L) in fresh extracts and after purification. Results are expressed as means ± SD of three sample replicates. *Significant statistical differences between purified and its fresh extract (Student's t-test, p<0.05).**

| Compounds (mg/L) | Fresh OLE | Purified OLE |
|---|---|---|
| **Phenolic Alcohols** | | |
| Hydroxytyrosol | 445.73±3.10 | 187.30±7.68* |
| Tyrosol | 12.09±2.58 | 5.83±0.16* |
| **Secoiridoids derivatives** | | |
| Oleuropein | 2173.52±110.80 | 679.53±12.73* |
| **Compounds (mg/L)** | **Fresh GTE** | **Purified GTE** |
| **Alkaloid** | | |
| Caffeine | 2232.52±208.16 | 1483.30±23.61* |
| **Catechins** | | |
| Gallocatechin | 2536.82±16.55 | 2215.18±59.39* |
| Epigallocatechin | 948.80±36.29 | 802.00±93.18 |
| Epigallocatechin gallate | 1829.27±172.21 | 109.32±3.27* |
| Gallocatechin gallate | 658.52±18.44 | 70.73±6.52* |

The purification of the phenolic compounds from the extracts and the elimination of the chlorophylls produced the loss of polyphenols. The total phenolic content was 8205,94 mg/L and 4680.52 mg/L for fresh GTE and purified GTE, respectively, while it was 2631.34 mg/L and 872.67 mg/L for fresh OLE and purified OLE, respectively. Therefore, the efficiency of the purification column was 57.03% and 33.16% for GTE and OLE, respectively. After these results, the main phenolic compounds and caffeine in fresh GTE were quantified individually by HPLC (table 3): gallocatechin (2536.82 mg/L), caffeine (2232.52 mg/L), epigallocatechin gallate (1829.27 mg/L), epigallocatechin (948.80 mg/L) and gallocatechin gallate (658.52 mg/L).

These results are in agreement with previous findings described by other researchers, such as Kang et al. [22], who identified them as majority compounds in the Korean GTE, with epigallocatechin gallate being the most concentrated. Perva-Uzunalić et al. [23] also identified these compounds as the most abundant in GTE, wherein EGCG represented 67.5% of the main catechins of green tea leaves.

The purification process generated a significant decrease in all these compounds found in the GTE (table 3). The most relevant decrease was observed in EGCG and gallocatechin gallate which was significantly different.

On the other hand, major phenolic compounds in OLE were oleuropein (2173.52 mg/L) and hydroxytyrosol (445.73 mg/L). Despite the fact that it was found at much lower concentration, tyrosol (12.09 mg/L) was also analysed, because it is an important biomolecule. In fact, the phenolic compounds identified represented more than 80% of the total compounds of the OLE [24]. In the same way, the purification of the extract caused a considerable decrease in the phenolic compounds analysed, with a significant reduction in oleuropein and hydroxytyrosol (table 3).

The elaboration process of the extracts was optimized for the preservation of phenolics compounds [25]. These compounds were water-soluble since we used aqueous phase to obtain the extracts. Methanol or hexane were rejected owing to its toxicity [26] with a view to future applications of the extracts in fields like medicine or agroindustry.

The loss in phenolic content during purification of the extracts is due to the fact that these compounds may be retained in the purification column and not fully recovered or that some of them may undergo transformations. This has been reported when extracts are subjected to a column purification process, whose phenolic compounds can react and suffer transformations into simpler compounds or interact with each other [27]. Specifically, this is what usually happens to gallates, which are transformed during similar processes into other compounds such as gallic acid. Another factor influencing the final efficiency of the purification process by a chromatographic column is the retention of compounds into the column. The final passage of the eluent through it to entrain the compounds of interest does not separate them all and, therefore, its final efficiency decreases.

This evident decrease in the phenolic content due to the process of purification and elimination of the chlorophylls may also generate a decrease in their bioactive properties, an undesirable side-effect in some cases, since the elimination of photosynthetic pigments is intended to enhance them.

The chemical data discussed in this research can be considered useful in providing information about the presence of major and minor compounds in olive leaves and green tea leaves. The amount of phenolic compounds is an important factor when the quality of extracts is assessed and because of the beneficial effects of these components on human health.

### *3.2. Antioxidant activity of extracts and effects of chlorophylls*

The antioxidant activity of the phenolic compounds of green tea and olive leaves has been identified as one of their most important benefits, with catechins and oleuropein being the most powerful antioxidants in these groups [28, 8]. In order to verify that the possible loss of antioxidant activity is due to the loss of phenolic compounds during the purification process, the raw extract data were normalised to compare them with the purified extract. In case of GTE, the efficiency of the purification column was 57.03%, a figure proportional to the remaining antioxidant activity in the purified extract (no statistical differences). Then, it can be ruled out that chlorophylls play an important role in the antioxidant capabilities of GTE, which therefore indicates that the antioxidant activity appearing in the purified extract corresponds only to the effect exerted by phenolic compounds.

Results of antioxidant activity of fresh GTE, purified GTE and their different combinations (table 1) is showed in figure 1.

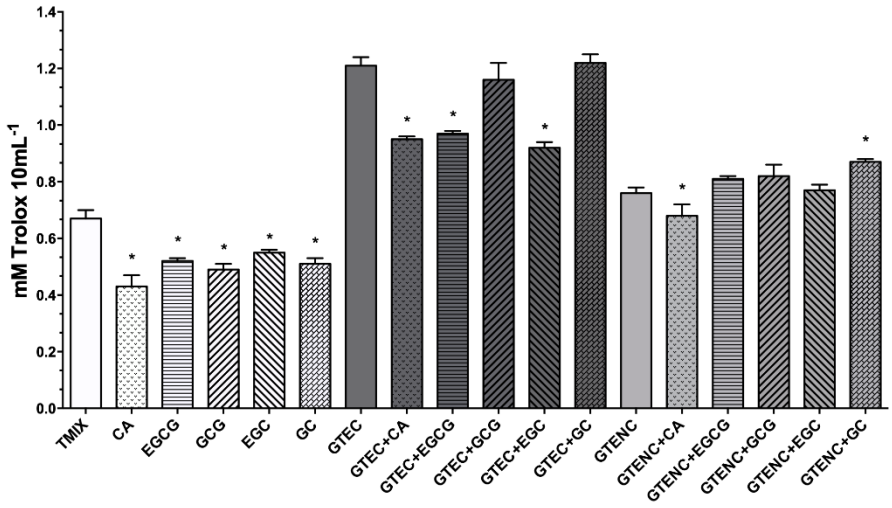


**Figure 1. Antioxidant activity of GTE showed as mM Trolox 10 mL$^{-1}$. Mixed pure majority phenolic compounds of GTE (TMIX), caffeine (CA), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epigallocatechin (EGC), gallocatechin (GC), GTE crude (GTEC), GTE crude with caffeine (GTEC + CA), GTE crude with epigallocatechin gallate (GTEC + EGCG), GTE crude with gallocatechin gallate (GTEC + GCG), GTE crude with epigallocatechin (GTEC + EGC), GTE crude with gallocatechin (GTEC + GC), purified GTE (GTENC), purified GTE with caffeine (GTENC + CA), purified GTE with epigallocatechin gallate (GTENC + EGCG), purified GTE with gallocatechin gallate (GTENC + GCG), purified GTE with epigallocatechin (GTENC + EGC) and purified GTE with gallocatechin (GTENC + GC) are analyzed. Results are showed as means ± SD. *Means show significant differences ($p<0.05$) by Tukey's multiple test between the doped extracts with their corresponding non-doped and between the individual compounds with the mix.**

As previously described in literature and corroborated by our own results, GTE has a high antioxidant capacity of 1.2 mM Trolox 10 mL$^{-1}$. All pure GTE compounds individually showed antioxidant activity, with EGC showing the highest antioxidant activity (significant differences; $p<0.05$). The mix of GTE compounds (TMIX) showed more antioxidant capacity than the individual compounds by themselves but it was not enough to confirm an accumulative or synergic effect between them. Numerous studies have shown the great antioxidant capacity of catechins, which are more effective in protecting the oxidative damage caused to DNA by free radicals than vitamin C [29], vitamin E, tocopherol and

carotenes [30]. Other researchers such as Lee et al. [31] stated that the total antioxidant capacity of tea is not related to a particular type of polyphenols, but to the combined activity of various antioxidants, including phenolic acids and polyphenols.

As for fresh extracts doped with pure compounds, the tendency is to maintain their antioxidant activity or a decreased onet with respect to undoped extract. This phenomenon is due to the pro-oxidant activity shown by phenolic compounds, which depends on the applied dose [5]. In fact, the increase of their concentration in the extract generates the appearance of pro-oxidant activity instead of increasing the antioxidant effect. The purified extract doped with the standards showed the same pattern of action as the non-purified extracts, with the exception of the gallocatechin-doped extract (GTENC+GC), which significantly increased its antioxidant activity. Similarly, saturation in the concentration of compounds added to the extract generated the appearance of pro-oxidant activity or no increase in antioxidant effect.

This experimental design is based on the results obtained by Wanasundara and Shahidi [18], which showed that GTE exhibited a pro-oxidant effect when applied to edible marine oils due to the catalytic effect of their chlorophyll, presenting an important antioxidant activity after pigment removal. These results indicate that the extract may present a negative dose-dependent matrix effect because, in some cases, the increase in the concentration of phenolic compounds causes a negative effect, thus decreasing the antioxidant capacity and in some cases even a pro-oxidant effect.

This pro-oxidant activity may be a positive feature for potential therapeutic applications against cancer. Therefore, consumption of green tea may induce oxidative stress, which may lead to ROS-mediated death of cancer cells or may eliminate ROS under conditions of high oxidative stress, thus preventing cell damage [32]. In addition, Azam et al. [19], stated that these pro-oxidant effects appear to be responsible for the induction of apoptosis in tumor cells, and may also induce activation of endogenous antioxidant systems in normal tissues that offer protection against tumor threat.

On the other hand, results of antioxidant activity of fresh OLE, purified OLE and its different combinations (table 2) is showed in figure 2.

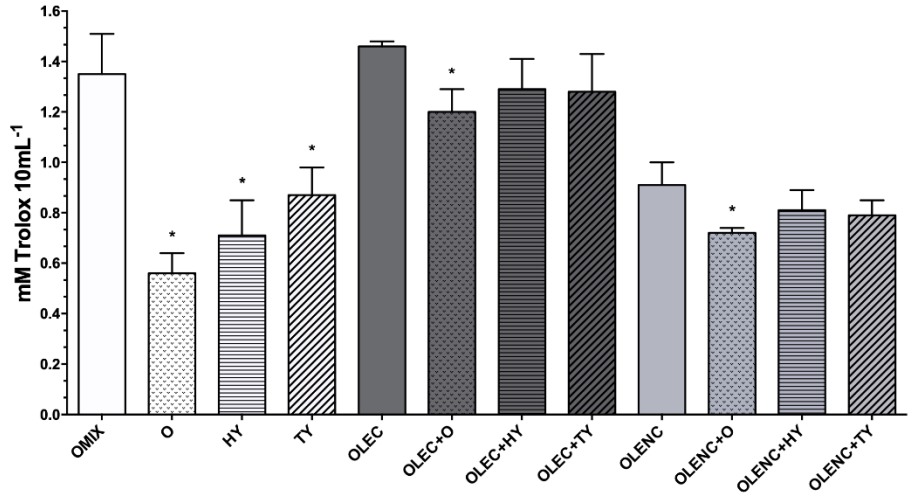


**Figure 2. Antioxidant activity of OLE showed as mM Trolox 10 $mL^{-1}$. Mixed pure majority phenolic compounds of OLE (OMIX), oleuropein (O), hidroxytyrosol (HY), tyrosol (TY), OLE crude (OLEC), OLE crude with oleuropein (OLEC + O), OLE crude with hydroxytyrosol (OLEC + HY), OLE crude with tyrosol (OLEC + TY), purified OLE (OLENC), purified OLE with oleuropein (OLENC + O), purified OLE with hydroxytyrosol (OLENC + HY), purified OLE with tyrosol (OLENC + TY) are analyzed. Results are showed as means ± SD. *Means show significant differences ($p<0.05$) by Tukey's multiple test between the doped extracts with their corresponding non-doped and between the individual compounds with the mix.**

Normalized data from fresh OLE reflected that the decrease in the antioxidant activity of the purified extract was not proportional (lower) to the reduction in phenolic content due to the purification process (statistical differences), thereby indicating that chlorophylls are not important for the antioxidant activity of OLE. This piece of data corroborated that a superior concentration of phenolic compounds generates a pro-oxidant activity [5]. Fresh OLE presented high antioxidant activity with value of 1.46 mmol Trolox 10 $mL^{-1}$. In fact, the OLE used herein had much higher antioxidant activity, 15-fold higher, than other foods such as virgin olive oil [25]. The OLE showed a similar behaviour to the GTE. Pure standard compounds had an important antioxidant activity when used individually: tyrosol had the greatest capacity and oleuropein the lowest. This activity increased when the compounds were put together, but the mixture (OMIX) did not show a synergic effect because this activity was lower than the sum of their individual activities. In addition, both the fresh and the purified OLE displayed the same behaviour. All the doped extracts exhibited decrease antioxidant activity but only the oleuropein-doped extracts (OLEC+O and OLENC+O) presented statistically significant differences. These reductions support the previous results observed on the dose-dependent pro-oxidant activity of phenolic compounds.

The preservation of phenolic compounds in OLE is desirable for its use for preventive and/or therapeutic purposes due to their protective effect against oxidative damage in human red blood cells [33]. Likewise, some authors reported the anticancer potential shown by olive phenolic compounds like oleuropein [34]. Not only the oleuropein displayed cytotoxic activity, but also hydroxytyrosol induce the death of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis [9]. Finally, other studies corroborated that polyphenolic antioxidants exhibited a dose-dependent toxicity against human promyelocytic leukemia cells (HL- 60), which was accompanied by malondialdehyde formation [35]. For these reasons, the goal of this study was to explore in depth the relationship between different molecules that compose the extracts and their antioxidant and anti-cancer activity.

### *3.3. Cytotoxic activity of extracts and chlorophylls effects*

There is a lot of literature describing the anti-cancer or cytotoxic activity of plant extracts and phytochemicals contained in them. However, it is necessary to deepen and investigate the mechanisms of action of these molecules, the influence of others present in the extracts, and the interaction between them.

Figure 3 shows the results of cytotoxic activities of the different GTE assayed (table 1) against human promyelocytic leukemia HL-60 cell line.

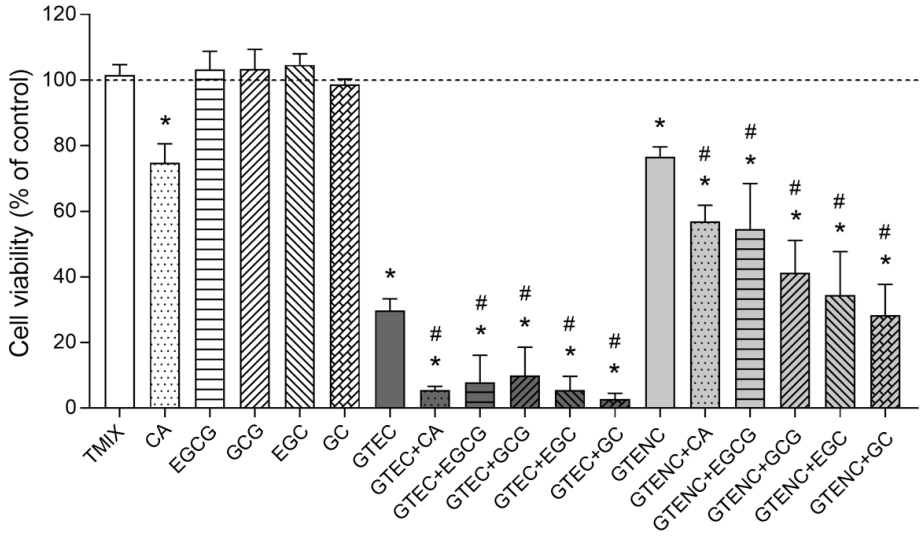


**Figure 3. Antiproliferative activity of mixed pure majority phenolic compounds of GTE (TMIX), caffeine (CA), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epigallocatechin (EGC), gallocatechin (GC), GTE crude (GTEC), GTE crude with caffeine (GTEC + CA), GTE crude with epigallocatechin gallate (GTEC + EGCG), GTE crude with gallocatechin gallate (GTEC + GCG), GTE crude with epigallocatechin (GTEC + EGC), GTE crude with gallocatechin (GTEC + GC), purified GTE (GTENC), purified GTE with caffeine (GTENC + CA), purified GTE with epigallocatechin gallate (GTENC + EGCG), purified GTE with gallocatechin gallate (GTENC + GCG), purified GTE with epigallocatechin (GTENC + EGC), and purified GTE with gallocatechin (GTENC + GC). Results are showed as means ± SD. *Means show significant differences against the control (100 % of viability) by Tukey's multiple test ($p<0.05$). #Means show significant differences between the doped extract and its corresponding non-doped extract by Tukey's multiple test ($p<0.05$).**

Normalized data of fresh GTE showed that the elimination of chlorophylls produced a negative effect on the cytotoxic potential of the fresh GTE as cytotoxic activity was decreased by 50% after the purification process (statistically significant). These results, in contraposition of antioxidant capacity of GTE, indicate that the photosynthetic pigments have an important role in the extract's cytotoxic capacity.

As for the individual compounds of GTE, the only compound with cytotoxic activity was caffeine (74.85% of cell viability). The other compounds and the mix (TMIX) did not show activity, the latter probably due to a negative matrix effects of the other compounds on caffeine. The cytotoxicity of caffeine had been described by other authors, who affirmed that caffeine inhibited ATM and ATR kinases which leads to the disruption of multiple DNA damage-responsive cell cycle checkpoints and greatly sensitized tumor cells to antitumor agents [36]. Caffeine may also block repair of DNA lesions through a direct interference with DNA-PK activity and other DNA repair enzymes [37].

In addition, the doped extracts showed an important positive matrix effect. Both purified and non-purified doped extracts further increased their cytotoxicity as the concentration of pure compounds present in them also increased (statistically significant differences). The compound with the highest increase in cytotoxic activity was galocatechin (GC). This fact, together with the results showing that the individual compounds did not have cytotoxic capacity, reinforces the idea that catechins act by potentiating the cytotoxic activity presented by other molecules. As mentioned above, green tea catechins such as EGCG combined with anticancer drugs generate synergistic enhancement of

apoptosis, gene expression changes and anti-cancer effects [15]. Besides, Fujiki and Suganuma [38] confirmed that the combination of anti-cancer drugs with green tea catechins synergistically induces apoptosis of human cancer cells, inhibits tumor formation in mice, and enhances inhibition of tumor growth in xenograft mouse models.

On the other hand, figure 4 shows the results of cytotoxic activities of the different OLE assayed (table 2).

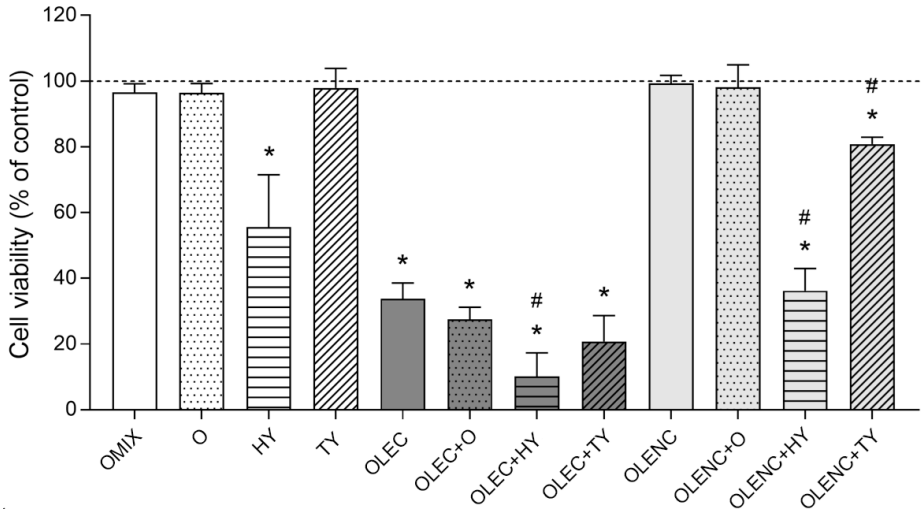


**Figure 4. Antiproliferative activity of mixed pure majority phenolic compounds of OLE (OMIX), oleuropein (O), hidroxytyrosol (HY), tyrosol (TY), OLE crude (OLEC), OLE crude with oleuropein (OLEC + O), OLE crude with hydroxytyrosol (OLEC + HY), OLE crude with tyrosol (OLEC + TY), purified OLE (OLENC), purified OLE with oleuropein (OLENC + O), purified OLE with hydroxytyrosol (OLENC + HY), and purified OLE with tyrosol (OLENC + TY). Results are showed as means ± SD. *Means show significant differences against the control (100 % of viability) by Tukey's multiple test ($p<0.05$). #Means show significant differences between the doped extract and its corresponding non-doped extract by Tukey's multiple test ($p<0.05$).**

Like in fresh GTE, normalized data of fresh OLE demonstrated that the elimination of chlorophylls generated a marked decrease in its cytotoxic activity due to the purification process. The purification process allowed to recover 33% of the phenolic compounds found in the extract with the consequent elimination of chlorophylls. This fact, together with the results already described, indicates that chlorophylls play a major role in the anti-cancer activity of OLE. The importance of the chlorophylls in chemoprevention has been previously described. Thus, chlorophyll and chlorophyllin can form complexes with certain chemicals that cause cancer such as aflatoxin-B1. These complexes may interfere with gastrointestinal absorption of potential carcinogens, and the amounts of carcinogenic substances in susceptible tissues may be reduced [17]. Also, this molecule is credited with the ability to prevent cancer by means of different mechanisms including its antioxidant and antimutagenic activity, mutagen trapping, modulation of xenobiotic metabolism, and induction of apoptosis [39]. Although, the direct action of chlorophylls against cancer cells have not been described yet, it is clear the importance of these compounds for future anti-cancer therapies.

In addition, with regard to the OLE pure compounds, only the hydroxytyrosol showed a cytotoxic activity (55.56% of cell viability). The OMIX did not show cytotoxic activity probably for the same reason as TMIX, i.e., the other compounds produced a negative matrix effect on hydroxytyrosol.

Finally, the doped extracts showed the same behaviour (both fresh and purified) in so that oleuropein and tyrosol generated a slight increase in cytotoxic capacity while hydroxytyrosol caused a large increase on such activity (statistically significant). The anti-cancer capacity of hydroxytyrosol is well known, although the mechanisms of action are not completely clear. Several studies demonstrated that this phenolic compound is a potent antioxidant phenol and also possesses antiproliferative, pro-apoptotic, as well as anti-inflammatory activities. These activities have the potential to counteract specific cancer hallmarks, thus representing the expectant biological activities underlying the anti-tumor properties of hydroxytyrosol [40]. It has been also reported that hydroxytyrosol has a significantly protective effect against UVB-induced oxidative DNA damage due to its antioxidant activity [41]. Moreover, it has been shown that hydroxytyrosol protects against other different cancer cell types, such as those derived from gut [42], blood [43], prostate [44], and breast [45].

Given the results obtained in this study and those from previous experiments which remark the importance of chlorophylls, one can speculate that there are substantial possibilities for using both extracts (GTE and OLE) as potential tools in future human anti-cancer research and therapy. Nevertheless, it is necessary to further investigate on the mechanism of action of these molecules and the interaction effect between them to potentiate their activity.

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