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Full Length Research Paper

Free radical scavenging and cytotoxic activity of five commercial standardized extracts (red wine, green tea, pine bark, polygonum and pomegranate)

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The objective of extract standardization is to obtain optimum and consistent quality of herbal preparations with defined components. This paper presents the radical scavenging effect of extracts against DPPH free radical (2,2-diphenyl-1-picrylhydrazyl) and cytotoxic activity *in vitro* on the cervical cancer cell line HeLa of five commercial standardized extracts rich in phenolic compounds: red wine (*Vitis vinifera*), green tea (*Camellia sinensis*), pine bark (*Pinus maritime*), polygonum (*Polygonum cuspidatum*), and pomegranate (*Punica granatum*). It shows radical scavenging activity in the following order, according to their median effective concentration (EC_{50}): *P. cuspidatum* 85 µg/ml, *C. sinensis* 11 µg/ml, *P. maritima* 7 µg/ml, *V. vinifera* 6 µg/ml, *P. granatum* 1 µg/ml and for positive control, vitamin E was 21 µg/ml. The cytotoxic activity, according to their half maximal inhibitory concentration (IC_{50}) was: *P. granatum* 22 µg/ml, *C. sinensis* 13.4 µg/ml, *P. cuspidatum* 12.8 µg/ml, *P. maritima* 11 µg/ml, and *V. vinifera* 7 µg/ml and for positive control, Triton X-100 was 3 µg/ml. Our results indicate that low concentrations of red wine, pomegranate, and pine bark extracts have high radical scavenging effect as well as cytotoxic activity on HeLa cells. Therefore, these extracts may be an important source for phytopharmaceuticals development.

Key words: Radical scavenging, cytotoxicity, HeLa cells, standardized extracts.

INTRODUCTION

Standardization extract can be defined as establishing reproducible pharmaceutical quality by comparing one product with established reference substances and defining minimum quantities of one or several compounds (Rodríguez-López, 2009). The extract standardization process is an indicator of quality and quantity that determines the quantitative and/or qualitative presence of a compound or group of compounds, in addition to presenting the extract in powdered form. A number of currently marketed standardized extracts are derived from a variety of plants and used as feedstock in nutritional supplement manufacturing. Most of these extracts are presented as phytochemical markers to type phenolic antioxidant compounds possessing OH^- groups (Lock de Ugaz, 1994). However, the growing range of chronic degenerative diseases as well as cancer, have increased the consumption of antioxidants in the world population to counter the high amounts of free radicals and possible development of cancer (Herber, 2007).

Cervical cancer (CC) remains a predominant cause of death in women worldwide, and the annual rate of cervical cancer remains high, usually greater than 20 cases per 100 000 women (Lewis, 2004). CC is a public health problem in Mexico and Latin America. It is the most common neoplastic disease and cause of death in women, and each year, 500 000 cases are diagnosed worldwide. In Mexico, it is the leading cause of cancer deaths in women older than 25 (Hidalgo-Martínez, 2006).

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There are reports that some antioxidants reduce certain types of cancer by inhibiting cell growth and preventing damage caused by free radicals in cancer, thus protecting healthy cells (Martínez-Flores et al., 2002; Gutiérrez-Maydata, 2002; Sagar et al., 2006; Hertog et al., 1993). The aim of this research is to assess the *in vitro* cytotoxic activity and free radical scavenging, or antioxidant activity, of five standardized extracts of phenolic compounds in the cervical cancer cell line, HeLa. These data would provide information to increase biological research of these extracts for possible applications in the development of phytopharmaceuticals.

MATERIALS AND METHODS

Reagents

The standardized extracts used were red wine (*V. vinifera*) to 30% polyphenols, green tea (*C. sinensis*) to 50% polyphenols, pine bark (*P. maritimus*) to 90% of pycnogenol, polygonum (*P. cuspidatum*) to 50% resveratrol, and pomegranate (*P. granatum*) to 40% ellagic acid. These extracts were kind gifts of Malabar Natural Products and Greenside S.A de C.V. The percentage composition was evaluated at each analysis and certificate for the compound or group of compounds were reported in each extract.

Cytotoxic effect with CellTiter Blue®

The adherent cervix adenocarcinoma cell line HeLa (ATCC: CCL-2) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 10 U/ml penicillin, and 10 µg/ml streptomycin. Cell cultures were used at 80% confluency, and trypan blue exclusion assays was performed to check cell viability. Cells were plated in 96 well micro plates at a density of 3 000 cells/well and incubated for 24 h in a humidified 37°C chamber with 5% CO_2. After 24 h, 100 µL of the extract solutions (0.1, 1, 10, 25, or 50 µg/ml, prepared in DMEM) were added to the cells and incubated for another 24 h under the same conditions. Subsequently, 50 µL was removed from each well as a positive control and was replaced with 50 µL Triton X-100. The micro titer plates were incubated for another 30 min, and 10 µL CellTiter Blue® reagent (Promega, USA) was added to each well. After 24 h, the absorbance was measured at 550 nm with a 630-nm differential filter using a micro plate reader (BIO-TEK, USA). The results are expressed as percentage viability by determining the half maximal inhibitory concentration (IC_{50}; inhibitory concentration that causes 50% inhibition of growth or cell death) (Williamson et al., 1998).

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging

The radical scavenging capacity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma Chemical Co., USA) was determined according to the following method (Garcia-Becerra et al., 2010): Solutions of different concentrations of extracts (0.1, 1, 10, 25 and 50 µg/ml) as well as of vitamin E for the positive control (Sigma Chemical Co.) were prepared. Methanol (CTR Scientific, Mexico) was added to a final volume of 100 µL. Next, 2 ml of a methanolic solution of DPPH (20 µg/ml) was added to each tube and incubated at room temperature for 20 min in the dark. The control was prepared without extract, and a blank was prepared with methanol for baseline correction. After incubation, the absorbance was measured at 517 nm in a spectrophotometer (Thermo Spectronic Genesys 20, USA). A decrease in the absorbance of the reaction mixture indicates radical scavenging capacity. DPPH radical inhibition caused by the extracts and vitamin E was calculated using the following formula:

$$Radical\ scavenging\ DPPH\ (\%) = \left[\frac{ABS_{t=0min} - ABS_{t=20min}}{ABS_{t=0min}}\right] x\ 100$$

Where, $ABS_{t = 0\ min}$ is the absorbance at 0 min and $ABS_{t= 20\ min}$ is the absorbance after 20 min of incubation. The results are expressed as the median effective concentration (EC_{50}), which is defined as the concentration at which a sample causes a 50% decrease of the initial concentration of DPPH.

Statistical analyses

Data are expressed as the means ± SD from three independent experiments. For cytotoxicity, the percent viability was compared with control wells in terms of IC_{50}, and radical scavenging activity by the EC_{50} using linear regression analyses. Statistical comparisons of results were performed using a Student's t- test and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

All extracts exhibited cytotoxic activity on HeLa cells. Table 1 shows the IC_{50} of cytotoxic activity and the EC_{50} of the radical scavenging activity found for each extract. The range of IC_{50} is presented at low concentrations in the range of 7 to 22 µg/ml, with the red wine extract the most active with an IC_{50} of 7 µg/ml. The highest cytotoxic effect was by the pomegranate extract at 22 µg/ml. Radical scavenging activity, expressed as EC_{50}, showed a wider range of 1 to 85 µg/ml. The most powerful antioxidant was pomegranate extract at 1 µg/ml and the highest EC_{50} was the polygonum extract at 85 µg/ml. There are published reports of biological activity about the five tested extracts in this paper as an antioxidant and cytotoxic agent in some cell lines. However, in these studies, all experiments were performed with extracts, fractions, or plant compounds. It is important to note that the advantage of standardized extracts is that they always contain a defined composition of the compound or group of compounds. Thus, this favors the formulated pharmaceutical, food, or cosmetic products, as standardization satisfies one of the requirements requested by the Pharmacopoeia.

There are reports from several authors regarding the antioxidant and cytotoxic activity of a methanol extract from pomegranate rind on the MCF-7 breast cancer cell line. By determining the amount of polyphenols using the Folin-Ciocalteu phenol reagent and using DPPH to determine the free radical scavenging activity, they determined that the component with the highest activity was ellagic acid. Ellagic acid possessed strong apoptotic activity and free radical scavenging activity (Dikmen et

Table 1. IC_{50} and EC_{50} values of standardized extracts.

Sample	IC_{50} values (µg/ml)	EC_{50} values (µg/ml)
Vitamin E (PC)	-	21
Triton X-100 (PC)	3	-
Red wine (*Vitis vinifera*)	7	6
Pine bark (*Pinus maritima*)	11	7
Polygonum (*Polygonum cuspidatum*)	12.8	85
Green tea (*Camellia sinensis*)	13.4	11
Pomegranate (*Punica granatum*)	22	1

PC, Positive control; -, Not detected.

al., 2011). Pomegranate has been tested on several cell lines derived from various tissues, including breast, colon, lung, and prostate. However, breast and prostate showed the most promising results (Adhami et al., 2009; Sturgeon and Ronnenberg, 2010; Koyama et al., 2010). A report found that in HeLa cells, pomegranate extract was one of the least effective extracts tested (McDougall et al., 2008). These results were corroborated in our investigation because pomegranate extract showed the lowest cytotoxic activity on HeLa cells with an IC_{50} of 22 µg/ml.

However, pomegranate had the highest free radical scavenging activity with an EC_{50} of 1 µg/ml. The positive control, vitamin E, had an EC_{50} of 21 µg/ml, and we determined that there was no significant difference between pomegranate extract and vitamin E (Student's t–test, $p < 0.05$).

The red wine extract used in this study showed 30% of polyphenolic compounds and showed a lower IC_{50} concentration of 7 µg/ml. This is the closest value to the positive control, Triton X-100, which had an IC_{50} of 3 µg/ml (Figure 1B). Therefore, this is a statistically significant difference. Triton X-100 is a non-ionic surfactant widely used in cell biology to permeate cell membranes. However, based on the results obtained here, it would be interesting to use a natural drug in the future with a similar action as Triton X-100 as chemotherapy to corroborate these effects and statistically evaluate the relationship. The objective of this study was a first attempt to evaluate standardized extracts and check for relevant activity using Triton X-100 as a positive control. There are reports in the literature showing that the polyphenols in red wine cause cell cycle arrest and induce apoptosis in the human lymphoblastic leukemia cell line, Jurkat (Sharif et al., 2010; Sharif et al., 2009).

In a previous study, the cytotoxic activity of polyphenols from red and white wine, including resveratrol, was investigated on the following cell lines: HeLa, MDA-MB-361, MDA-MB-453, and PBMCs. White wine polyphenols have a greater effect than red wine compounds, but both are active. In addition, the HeLa cell line was most susceptible to polifenoles (Matić et al., 2010). Our results confirm the cytotoxic effect for the red wine extract on HeLa cells, and it will be interesting to discover the mechanisms involved in the cytotoxic effect. As shown in Figure 1G, the red wine extract completely destroyed the cell monolayer, and because the method used to assess the cytotoxic effect did not involve washing, this gives a more accurate result and indicates no cells were lost due to washing, therefore, our protocol eliminates the possibility of lost cells, the *V. vinifera* extract showed an IC_{50} of 7 µg/ml.

Resveratrol (trans-3, 5, 4'-trihydroxystilbene) is a phytoalexin found in a variety of plants including grapes, red wine, berries, and nuts. However, the plant *P. cuspidatum* has become the main source for obtaining resveratrol. Resveratrol has several biological activities, such as antioxidant, anticoagulant, anti-inflammatory, cardioprotective, and anti-cancer properties (Mao et al., 2010; Pei-Chi et al., 2010; Abbott et al., 2010; Kramer and Wesierska-Gadek, 2009; Kraft et al., 2009; Lin et al., 2010). Pre-treatment with resveratrol followed by administration of taxol causes a synergist effect; however, the combination of these compounds considerably decreases the activity of taxol on bladder cancer cells (Mao et al., 2010).

Regarding cytotoxicity, a report has shown that resveratrol induces apoptosis and causes cell cycle arrest in the human hepatoma cell line Huh-7 (Pei Chi et al., 2010). Resveratrol also inhibits HeLa cell proliferation by blocking the S phase of the cell cycle and prevents angiogenesis by inhibiting the expression of vascular endothelial growth factor (VEGF) (Kraft et al., 2009). Previous study shows an association between the antioxidant effect and the antiproliferative effects by *P. cuspidatum* on DPPH in lung cancer cells and found compelling activity of scavenging of the DPPH free radical and a high antiproliferative effect (Lin et al., 2010). As shown in Table 1, the extract of polygonum had the highest EC_{50} of 85 µg/ml and an IC_{50} of 12.8 µg/ml (Figure 1E), and thus, our data are interesting and consistent with those previously reported, it is of great interest to continue studying resveratrol obtained from *P. cuspidatum*.

Green tea has been widely studied for its polyphenols

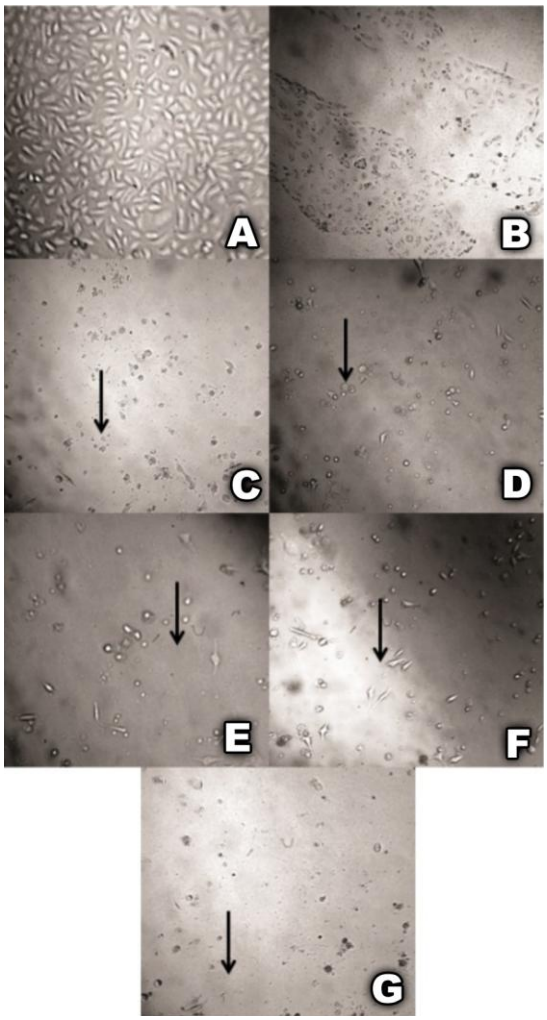

Figure 1. Standardized extracts effect on HeLa cells. Untreated cells (A) and cells after treatment with the positive control triton X-100 (B). The five evaluated extracts inhibit growth of HeLa cells besides producing morphological damage visible, it observes the disappearance of the monolayer, rounded cells, loss of morphological integrity and adhesion loss besides C (*P. granatum*), D (*C. sinensis*) E *(P. cuspidatum*), F (*P. maritima*), G (*V. vinifera*)].

because it has high antioxidant activity. In recent years, after this antioxidant was found to offer protection against the occurrence of cancer, studies evaluated the effects of green tea extracts on different cell lines. Several mechanisms of action were detected. Specifically in HeLa cells, pre-incubation of the ethanolic extract of green tea showed an IC_{50} of 256 µg/ml as assessed with the Kit II method of proliferation. Its mechanism of action is associated with inactivation of cytosolic thioredoxin reductase (Wang et al., 2008), and green tea also affects the function of VEGF and hypoxia-inducible 1α (HIF-1α) in HeLa cells (Zhang et al., 2006) and inhibits HeLa cell proliferation upon irradiation with a laser light (Sommer et al., 2010). Green tea extract also decreases cell proliferation of the breast cancer cell line MDA-MB231 (Sartippour et al., 2002). In the previous investigations, polyphenols inhibited proliferation and cell death.

The results from this study are consistent with reports regarding the cytotoxic effect of green tea polyphenols on HeLa cells, particularly because the IC_{50} was 13.4 µg/ml and the scavenging effect of free radicals showed an EC_{50} of 11 µg/ml. This shows better activity than the positive control (vitamin E) used in this trial with no statistically difference (Student's t-test, $p < 0.05$). This particular result can be compared to a report in which a mixture of extracts from several plants was used, including green tea, and a synergistic effect was determined to enhance the antioxidant effect (Jain et al., 2011) of the green tea flowers that have strong antioxidant activity of 11.6 µg/ml as measured by the DPPH method (Yang et al., 2007).

P. massoniana and *P. pinaster* bark have been widely studied, and an extract of *P. maritima* was standardized to 90% in pycnogenol (monomeric and oligomeric polyphenols). Some clinical trials have demonstrated the efficacy of pycnogenol in situations such as dysmenorrhea, seminal fluid analysis, attention deficit disorder, venous insufficiency, and microangiopathy (Navarro-Moll et al., 2010; Shen et al., 2010). This suggests the bark has important biological actions. A report showed that flavonoid-rich extracts of *P. massoniana* have cytotoxic activity on HeLa and MDA-MB-23133 cells and that they inhibit proliferation, induce apoptosis, and cause cell cycle arrest in HeLa cells (Ma et al., 2008). Similarly, we have evaluated the antioxidant effect of extracts of *P. pinaster* by DPPH, ABTS [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], and HNTTM [tris (2, 4, 6-trichloro-3, 5-dinitrophenyl) methyl] with excellent results (Touriño et al., 2005), and our findings add to the information regarding the biological activity of extracts of the genus *Pinus*. The extract used in *P. maritima* showed good cytotoxic activity (IC_{50} of 11 µg/ml) and free radical scavenging activity (EC_{50} of 7µg/ml) with no statistically significant difference compared to vitamin E (Student's t-test, $p < 0.05$). Pycnogenol and *Pinus* extracts have a wide range of biological activities, including antioxidant and free radical scavenging activity, anti-inflammatory and cardioprotective activity, prevention of allergy, asthma, and high cholesterol, improvement in venous and menstrual disorders and attention deficit disorder, as well as antimicrobial and antiviral activity (Iravani et al., 2011). These effects are on HeLa cells found in this investigation in addition to the cytotoxic effect.

Conclusion

The five extracts tested in this work showed cytotoxic activity in HeLa cells in the following order, according to

their IC_{50}: Pomegranate (*P. granatum*) 22 µg/ml, green tea (*C. sinensis*) 13.4 µg/ml, polygonum (*P. cuspidatum*) 12.8 µg/ml, pine bark (*P. maritima*) 11 µg/ml, and red wine (*V. vinifera*) 7 µg/ml. The extracts tested in this work showed free radical scavenging activity measured by the DPPH method without statistically significant differences compared to vitamin E except the polygonum extract. The extracts were as follows, according to their EC_{50}: polygonum (*P. cuspidatum*) 85 µg/ml, green tea (*C. sinensis*) 11 µg/ml, pine bark (*P. maritima*) 7 µg/ml, red wine (*V. vinifera*) 6 µg/ml, and pomegranate (*P. granatum*) 1 µg/ml. The antioxidant activity of phenolic compounds and their cytotoxic effects are closely related (Romero-Cerecero et al., 2011; Lin et al., 2010), and our results support this.

Standardized extracts can be directly used in *in vitro* and *in vivo* assays as well as clinical trials (Romero-Cerecero et al., 2011; Hernández-Pérez and Herrera-Arellano, 2011). These compounds have standardized which can use as quality markers, ensuring the composition of assets present.

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