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Research Article

Polyphenolic extracts of walnut (*Juglans regia*) green husk containing juglone inhibit the growth of HL-60 cells and induce apoptosis

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ABSTRACT

Background: Juglone is a naphthoquinone currently obtained by chemical synthesis with biological activities including antitumor activity. Additionally, juglone is present in the green husk of walnut, which suggests evaluating the effect of GH extracts on carcinogenic cell lines.
Results: Walnut green husk ethanolic extract was obtained as 169.1 mg $_{\text{juglone}}$/100 g $_{\text{Green Husk}}$ and antioxidant activity (ORAC) of 44,920 μmol $_{\text{Trolox Equivalent}}$/100 g DW $_{\text{Green Husk}}$. At 1 μM juglone in HL-60 cell culture, green husk extract showed an antiproliferative effect, but pure juglone did not; under these conditions, normal fibroblast cells were not affected. A dose-dependent effect on mitochondrial membrane potential loss was observed. Apoptosis of HL-60 was detected at 10 μM juglone. Despite high ORAC values, neither purified juglone nor the extract showed protective effects on HL-60 cells under oxidative conditions.
Conclusions: Green husk extract generates an antiproliferative effect in HL-60 cells, which is related to an induction of the early stages of apoptosis and a loss of mitochondrial membrane potential. The normal cells were not affected when juglone is present at concentrations of 1 μM, while at higher concentrations, there is loss of viability of both cancerous and healthy cells.
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1. Introduction

Juglone is a naphthoquinone (5-hidroxy-1,4-naphthoquinone) present in plants of the Juglandaceae family and is found in various parts of different species of walnut (*Juglans regia* and *Juglans nigra*), such as husks, leaves, roots and root hairs, and pericarp [1,2], along with other polyphenols. These different parts of the walnut tree have been used as bases for tinctures and, in natural medicine, as part of alcoholic beverages with beneficial health effects such as anti-inflammatory, antibacterial, antifungal, and anticarcinogenic effects [3]. Additionally, an allelopathic effect of the juglone has been established, and thus, it is recognized as an effective herbicide because it inhibits metabolic enzymes with a consequent inhibition of photosynthesis [4].

In particular, the green husk (exocarp and mesocarp of the walnut), which is a crop residue of this fruit, is recognized as a potential source of polyphenol compounds. Thirteen different phenolic compounds have been identified in the walnut, principally gallic acid, vinyl acid, ellagic acid, protocatechuic acid, catechin, epicatechin, myricetin, and juglone; the last represents 30% of the phenolic compounds present in the green husk [2,5,6]. The juglone content in the green husk varies according to the moment in which the harvest is carried out; however, juglone is always the only phenolic compound with the greatest presence, as reported by Stampar et al. [2]. The phenolic compounds present in the walnut have been recognized for having several effects, which can be beneficial to health, such as effectively inhibiting low-density lipoprotein (LDL) oxidation in plasma [7]. In relation to other beneficial effects, several studies have demonstrated the anticancer activity of the above-mentioned phenolic compounds and, in particular, the antiproliferative capacity of pure juglone on different oncogenic cell lines and normal cells [8,9,10,11,12]. These latter studies were carried out using a commercial purified product where the juglone content is approximately 95-98%. These studies

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have not evaluated the potential effects of natural extracts that contain juglone and other phenolic compounds and, in some cases, have not considered the possible effects of these concentrations of juglone on normal cells in a potential therapeutic application.

Accordingly, this work evaluates the biological activity of a walnut green husk extract containing juglone and other phenolic compounds through a study of antiproliferative and antioxidant effects on human promyelocytic leukaemia cells HL-60, using healthy fibroblast cells as a control in the antiproliferative study. The extract is evaluated by comparing it with a commercial purified juglone product and a standard antioxidant of known biological activity as control.

2. Material and methods

2.1. Reagents and Cell Lines

The reagents and standards used were of analytical grade. Folin-Ciocalteu phenol reagent, sodium hydroxide, and sodium carbonate were purchased from Merck (Germany). Trolox, gallic acid, and other reagents were from Sigma-Aldrich (USA).

The human promyelocytic leukaemia HL-60 cell line has been widely used to study aspects of oxidative stress. This cell line was obtained from Sigma-Aldrich (98070106) (USA). A primary fibroblast culture was supplied by the Cell Culture Laboratory of Pharmacy Faculty (Universidad de Valparaíso, Chile).

2.2. Walnut green husk

The walnut green husk was obtained 170 days after flowering from a walnut plantation in the Libertador General Bernardo O'Higgins Region (33°56'00"S 71°50'00"W; Chile). The walnut green husk had a moisture content of 85%. At the time of walnut harvest, the sample was stored frozen and then lyophilized using an Ilshin freeze-dryer (-53°C; 50 mTorr). Then, the lyophilized sample was homogenized using an IKA A 10 Basic mill and vacuum packaged until its use. The walnut green husk was characterized, obtaining a dry basis composition of 18.67% ash, 17.72% of protein, 6.91% of lipids, 48.42% crude fiber, and 8.28% non-nitrogen extract.

2.3. Walnut green husk extract production

Solvent extraction with ethanol or methanol was performed at a temperature of 40°C for 6 hours using a solid-to-liquid ratio of 1:20 [13]. The walnut green husk was used either raw (green husk natural: GHN) or previously lyophilized (green husk lyophilized: GHL). The supernatants were filtered using a 0.45 μm mesh to obtain walnut green husk extracts, and the content of total phenolic compounds, antioxidant activity (DPPH and ORAC), and juglone content were determined for each extract.

2.4. Juglone determination by HPLC

The content of juglone in the walnut green husk extracts was determined by high-performance liquid chromatography (HPLC) using a photo diode array (PDA) detector in PerkinElmer 200 series equipment. The equipment was controlled using TotalChrome® Chromatography Data System (CDS) software. A Kinetex Evo® C18 100 A 5 μm (250 mm x 4.6 mm) column was used. The oven temperature was set to 25°C. A gradient of solvents was used as the mobile phase: solvent A was 2.0% acetic acid in aqueous solution and solvent B was 0.5% acetic acid in aqueous solution and acetonitrile (1:1 ratio). For the detection of juglone, the gradient used was as follows: 90% A to 40% A (50 min), 45% A to 0% A (10 min), and 0% A to 90% A (5 min). The feed flow was 1.0 mL/min, and the sample volume injected was 20 μL. The total measurement time was 65 min. Juglone detection was done at 280 nm.

2.5. Total phenolic content determination

The total phenolic content (TPC) of the walnut green husk extracts was determined by the Folin-Ciocalteu reagent method [14]. A total of 3.75 mL of distilled water, 0.5 mL of extract sample, 0.25 mL of Folin-Ciocalteu reagent diluted 1:1 (v/v) with distilled water were mixed, and, after waiting 1 minute, 0.5 mL of sodium carbonate solution (10% w/v) were added. The absorbance was measured at 765 nm after 1 h of reaction at room temperature. The calibration curve was constructed using gallic acid as a standard (0-100 mg/L). The results were expressed as gallic acid equivalents (GAE) per 100 g of walnut green husk in dry weight (DW).

2.6. Antioxidant capacity determination by ORAC method

The capacity of the walnut green husk extracts to absorb peroxyl free radicals was assayed by the ORAC method [14]. The extract samples, a Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard and AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride), were prepared with phosphate buffer (pH 7.4). Twenty microliters of extract or phosphate buffer sample (blank) and 200 μL of fluorescein (1.5 mM) were added to each well in a 96-well opaque plate. The plate was incubated at 37°C for 10 min. Then, 75 μL of APPH (79.7 mM) was added to each well. Fluorescence was recorded every 1 min for 1 h at 37°C using excitation and emission wavelengths of 485 and 538 nm, respectively. The antioxidant capacity was calculated as Trolox equivalents using the relative area of the sample curves rather than a calibration curve. The calibration curve was constructed using Trolox as the calibration standard (20-90 μM).

2.7. Antioxidant capacity determination by DPPH method

The free radical scavenging activity of the walnut green husk extracts was determined by the DPPH method [14]. Each extract sample (50 μL) was mixed with 2 mL of a methanolic solution of DPPH (0.036 mM). The absorbance was measured at 515 nm after 16 min of reaction at room temperature. Methanol was used as blank. The antioxidant capability was expressed as the percentage of discoloration of the DPPH solution according to the following equation:

$$\%inhibition = \frac{A_0 - A_{16}}{A_0} \quad (1)$$

where,

A_o = absorbance at zero time

A_{16} = absorbance after 16 min of reaction.

In addition, DPPH determination was done using Trolox as the calibration standard. The antioxidant capacity was calculated as mg of Trolox equivalents per 100 g DW of GHL.

2.8. Cell culture, growth conditions, and treatments

The HL-60 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 g/L D-glucose, and 2 mM L-glutamine. Cells were grown in a CO_2 incubator (Forma Scientific, USA) at 37°C in an atmosphere of 5% CO_2 with 95% humidity. HL-60 cells were suspended at 2.5 x 10^5 cells/mL and cultured in 12-well plates (Orange Scientific, Belgium).

To assess the antiproliferative and antioxidant effects of the extract, cells were incubated for 24 h at 37°C under the above-mentioned conditions before the treatments that established the effects of the walnut green husk extract. Controlled trials in the proliferation experiment were performed without disturbance. The assays contained final concentrations of 1 and 10 μM juglone in the cultures by using either purified commercial juglone (control cultures) and

walnut green husk extract (assay cultures), respectively. The juglone concentration was selected according to the studies conducted by Paulsen and Ljungman [11] and Park et al. [15], who reported that 10 μM pure juglone was cytotoxic to fibroblasts, but extracts of walnut leaf, which contained approximately 1 μM juglone, did not affect the cells.

The same assays were performed under oxidative stress conditions using 0.12 mM H_2O_2 as an oxidizing solution [16].

The cells were exposed to the treatments for 24 h. Then, cell counting (viable and not) was performed. Mitochondrial membrane potential ($\Delta\Psi m$) and early apoptosis were determined by flow cytometry.

2.9. Cell viability determination

Counting of live and dead cells was performed using a hemocytometer, and cell viability was determined by the cell exclusion method using 0.2% trypan blue solution.

2.10. Mitochondrial membrane potential loss

A cell sample was used to detect the loss of mitochondrial membrane potential ($\Delta\psi m$). HL-60 cells were incubated with 10 μg/mL JC-1 at 37°C. Loss of $\Delta\psi m$ was assayed by observing a shift in fluorescence emission from red (~590 nm) to green (~525 nm) by flow cytometry (Beckman Coulter Cytomics, FC 500) [17]. HL-60 cells were treated with 100 nM valinomycin as a positive control of membrane potential loss.

2.11. Early apoptosis determination

The determination of apoptosis signals was performed by obtaining a representative histogram of cell cycle phases. Propidium iodide was used as a detector of loss of cell membrane integrity, as it generates staining upon contact with cellular DNA. The percentage of apoptosis was determined according to the fraction of cells with sub-G1 DNA content. For this, $1 \cdot 10^6$ cells/mL were centrifuged and washed; the pellet was resuspended in 1 mL of cold 1X PBS; 3 mL of ethanol was added (dropwise) and vortexed (total time of approximately 1 h). Afterwards, the cells were washed twice with 1X PBS; then, 1 mL of propidium iodide staining solution was added to the cell pellet and homogenized; finally, 50 μL of stock solution of RNAse A was added and incubated at 4°C for 3 h. A flow cytometer (Beckman Coulter Cytomics, FC 500) was used to determine the fluorescence variation using an average speed and a total of 20,000 events per sample.

2.12. Statistical analyses

Extractions and analyses were performed in triplicate, and the data are presented as the mean value ± SD. GraphPad Prism 5 was used for statistical analyses. P-values < 0.05 indicated statistically significant differences between compared groups.

3. Results and Discussion

3.1. Extract production: Total phenolic and juglone content

The walnut green husk extracts were obtained by stirring at 200 rpm at a temperature of 40°C for 6 h using ethanol and methanol and two types of raw material, namely, natural green husk (GHN) and lyophilized green husk (GHL). As shown in Figure 1, ethanol produced the best results ($P < 0.01$) for TPC and juglone extraction, as well as the antioxidant capacity of the extracts, except for GHL juglone content, where ethanol and methanol did not produce significant differences ($P > 0.05$). With regard to raw material, GHN showed better results than GHL, especially in TPC value, because the presence of water in the sample potentially facilitates the extraction of several phenolic compounds, as reported by several authors [13,18]. For subsequent tests, the extract obtained with ethanol from the lyophilized samples was used because of the lower toxicity of ethanol. Lyophilized samples were used because they are shelf-stable and require little storage space, hence benefiting the productivity of the extraction process at the industrial level.

Under these conditions, the concentration of juglone obtained was 120.7 ± 12.1 mg/L, while the total polyphenol content was 1,862.9 ± 72.4 mg GAE/100 g DW (1,330.7 mg GAE/L). The amount of juglone extracted from GHL was 169.1 mg/100 g DW, which is equivalent to 25.36 mg/100 g of fresh walnut green husk, considering a moisture content of 85% (determined in this work). This value is in the same order of magnitude as that reported by Cosmulescu et al. [1], i.e., between 20.56 and 42.78 mg/100 g from different varieties of walnut green husk. Stampar et al. [2] report a great variability in the presence of juglone on GHL, which is dependent on the sampling time, with values between 218 and 1404 mg/100 g DW. The content of total phenolic compounds was like the content of phenolics (1,526 mg/100 g DW as the sum of individual phenolic compounds) reported by Stampar et al. [2]. With regard to the effect of the type of solvent used for the extraction, it was possible to obtain better performance with ethanol extracts, mainly in the ORAC analysis and GHN juglone content; this fact is mainly due to the different solubilities of naphthoquinones in organic solvents and the relationship between the solubilities of these compounds and the processing temperature. For example, Song et al. [19] indicated that 2-methyl-1,4-naphthoquinone is 48.4% more soluble in ethanol than in methanol at 26°C, whereas at a temperature of 40°C (as used in this work), this difference is 98.02% in favor of ethanol.

3.2. Antioxidant capacity of the extract

The antioxidant capacity of the extract was determined by two methods: DPPH and ORAC. For the DPPH free radical scavenging method, Bakkalbasi et al. [20] reported that walnut methanolic extracts were capable of inhibiting the free radical DPPH by 35.8% to 85.2%, depending on the fruit variety, when the extract recovered was diluted 5 times. In the current work, when the extract obtained was diluted at least 10 times, a 30% inhibition was achieved, which indicates that the extraction processes were more efficient than those previously reported and yielded an extract rich in compounds capable of scavenging the DPPH free radical. In terms of trolox equivalents, the extract was obtained with ethanol as GHL shows an activity of 2663.2 ± 154.6 mg Trolox Equivalent/100 g_{GH} DW. On the other hand, when measuring antioxidant activity by means of the absorption capacity of the oxygen radical (ORAC), walnut green husk extract showed an activity of 44,920 ORAC units (μmol Trolox Equivalent/100 g_{GH} DW). This antioxidant activity is equivalent to that of species such as basil and cacao, with reported antioxidant capacities between 55,000 and 61,600 ORAC units [21] and those higher than those reported for some so-called “superfruits” such as berries, which present values between 16,000 and 40,000 ORAC units [21] and even higher than the values of antioxidant activity found in walnut meat (approximately 13,300 μmol Trolox Equivalent/100 g_{GH} DW). This result is in agreement with the work of Laroze et al. [13], who indicated that the external parts of fruits and vegetables and other lignocellulosic parts such as skins, husks, and seeds have a high content of antioxidant phenolic compounds, surpassing those detected in the corresponding internal fractions.

3.3. Cellular Growth

The effect of walnut green husk extract under nonoxidizing conditions on the growth of the HL-60 cell line was evaluated at concentrations equivalent to 1 and 10 μM juglone. The results were

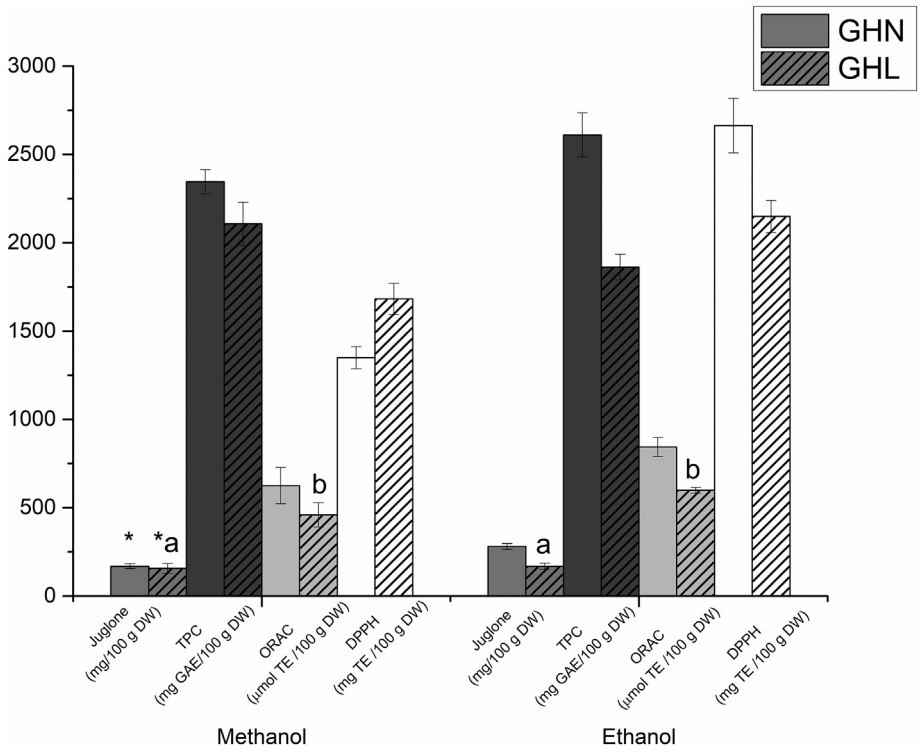

Figure 1. Effects of green husk condition and extraction solvent on the recovery of total phenolic compounds and juglone and the antioxidant capacity of the extracts. Extraction: 40°C, 6 h, solid/solvent ratio 1/20. GHN: green husk natural; GHL: green husk lyophilized. The same letter means no significant differences between solvents used for extraction (ethanol or methanol); the same number of asterisks means no significant differences between raw materials (GHN or GHL).

also compared to assays performed under the same concentrations of commercial purified juglone and a negative control. In addition, because the extract was obtained through ethanol solvent extraction, it was evaluated whether the final ethanol concentration in the culture medium (0.14% v/v) had any effect on the cells. It was established that this ethanol condition did not have a cytotoxic effect compared to the control at optimum culture conditions, obtaining similar growth levels ($12.2 \cdot 10^5$ cells/mL) and viability (98%) 24 hours after treatment (data not shown).

Regarding the effect of the commercial purified juglone on the growth of HL-60 cells, it can be observed in Figure 2a that the lowest concentration used (1 µM) did not produce an antiproliferative or cytotoxic effect. On the other hand, when using juglone at 10 µM, a marked antiproliferative effect and a decrease in cell viability of 12% were observed, suggesting a mild cytotoxic effect.

The effects of walnut green husk extract on the growth of HL-60 cells at concentrations of 1 and 10 µM juglone are also shown in Figure 2a**.** The lower concentration of extract generated a significant decrease in the number of living cells from 12.2 $x \cdot 10^5$ (control) to 8.3 $x \cdot 10^5$ cells/mL, whereas the extract at the 10 µM juglone level showed an antiproliferative effect, presenting a cellular concentration of 3.8 $x \cdot 10^5$ cells/mL after 24 h (reduced from 4.2 $x \cdot 10^5$ cells/mL at the start of the treatment). In addition, when the extract with 1 µM of juglone was used, the viability of the culture did not decrease significantly (from 98% to 97.3%), while in the case of 10 µM juglone extract, the viability dropped from 98% to 83.3%.

Whether using purified juglone or green husk extract, if a 10 µM concentration of either naphthoquinone was used, an antiproliferative effect could be obtained. One of the mechanisms potentially involved is the rapid reduction of this quinone, creating a semi-quinone radical

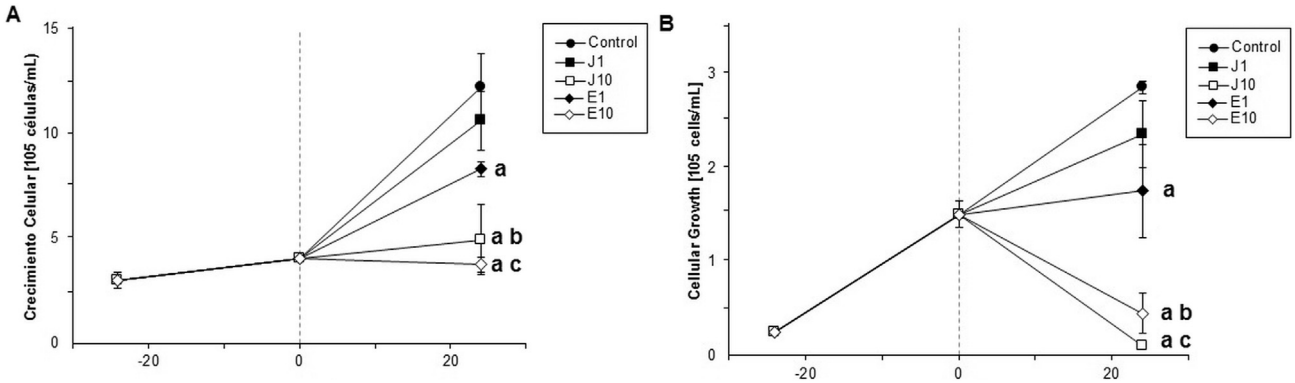

Figure 2. Effects of juglone-containing walnut green husk extract and commercial purified juglone on the cell growth of A) HL-60 and B) fibroblasts under nonoxidative conditions. Control: cultivation under optimum conditions; J1: culture with 1 µM purified juglone; J10: culture with 10 µM purified juglone; E1: culture with walnut green husk extract with final concentration of 1 µM juglone; E10: culture with walnut green husk extract with a final concentration of 10 µM juglone. **a**: Significant differences when compared with Control; **b**: significant differences when compared with J1; **c**: Significant differences when compared with E1.

that participates in the redox cycle of the mitochondria, thereby causing a rapid reduction of molecular oxygen toward the formation of superoxide radical anions, hydrogen peroxide, and hydroxyl radicals [22]. In other words, juglone, on the one hand, can interact with the redox cycle of the cell, generating reactive oxygen species (ROS) that damage the DNA of the cells [23] and provoke the antiproliferative effect. On the other hand, this effect was greater and even slightly cytotoxic when walnut green husk extract was used, which suggests a synergic effect with other compounds distinct from juglone but also present in the extract.

To establish the potential application of this type of compound in anticancer therapies, it is necessary to study their effects on the cellular viability of a normal or healthy cell line. Vardhini et al. [12] reported that concentrations higher than 0.32 mM of pure juglone had a cytotoxic effect on human lymphocytic cells. In the case of peripheral blood mononuclear cells (PBMC), a decrease of 50% viable cells (IC50) was observed when the concentration of pure juglone exceeded 28.7 μM [24], whereas for fibroblasts, Paulsen and Ljungman [11] showed that a concentration of 10 μM juglone was cytotoxic. Extracts of Japanese walnut leaves (*Juglans mandshurica*) do not show a cytotoxic effect on HS68 fibroblasts when the extracts are used in concentrations between 50 and 400 μg/mL [15], equivalent to 0.12 and 0.96 μM juglone [25]. Accordingly, Figure 2b shows the effect of purified juglone as well as walnut green husk extract on fibroblasts. It is possible to observe that both the purified juglone and the extract, when applied at a concentration of 10 μM of naphthoquinone, generated a total loss of cell growth, which is considered cytotoxic for this type of cells; this result is in agreement with those of the aforementioned authors for juglone. This suggests that other compounds in the walnut green husk extract do not produce a protective effect for this cell line. On the other hand, when using purified juglone at a concentration of 1 μM, there was no significant difference in the concentration of viable cells compared to the control culture after 24 h; when using the extract at the same concentration of juglone, a decrease in viable cells was observed. However, this latter variation was still not significant, which was consistent with the results presented for HS68 cells by Park *et al.* [15]. These results potentially indicate that the nonjuglone antioxidant compounds present in green husk extract do not generate an effect (positive or negative) on the viability or proliferation of fibroblasts.

3.4. Antioxidant effects

Figure 3 shows the antioxidant capacities of both walnut green husk extract and purified juglone on HL-60 cells, using a final culture concentration of 0.12 mM H_2O_2 as oxidant. In addition, a commercial antioxidant (Trolox) was used as an evaluation standard (positive control). As shown, using the oxidant under the above-mentioned conditions resulted in a complete loss of cell viability after 24 h of cultivation (cell growth $0.7 \cdot 10^5$ cells/mL; viability 11.3%). When using only the commercial antioxidant, there was no difference in growth compared to the control culture, suggesting that this compound was innocuous, with no antiproliferative or cytotoxic effects observed [16] and had a protective effect on the growth of HL-60 cells under oxidative culture conditions (cell growth $5.3 \cdot 10^5$ cells/mL; viability 74.9%). Pure juglone at 1 μM or 10 μM on HL-60 cells in the presence of the H_2O_2 oxidant had no protective effect against oxidative damage. The same behavior was observed when the walnut green husk extract was tested under the same conditions. This is probably due to the high reduction power of juglone, which would increase the production of ROS. At high concentrations, polyphenols with antioxidant activity can be transformed into pro-oxidants that generate a cytotoxic effect on cells. Juglone has been reported to cause ROS production in some cells such as gastric cancer cells [26,27], human lymphocytes [28], and human leukemia cells [29]. Consequently, juglone and other polyphenols contained in the extract do not have a protective effect on cell growth of HL-60 under oxidative conditions; on the contrary, a synergic effect of their cytotoxicity with oxidative stress is evidenced.

This effect, as well as the effect on the aforementioned redox cycle, was also reflected in the ΔΨm loss of HL-60 cells under oxidative and nonoxidative conditions.

3.5. Mitochondrial membrane potential loss and early apoptosis

Figure 4a shows the loss of mitochondrial membrane potential (ΔΨm) when the culture develops in an oxidizing or nonoxidizing medium.

In the presence of an oxidant, approximately 80% of cells lost their mitochondrial membrane potential, whereas this number was approximately 10% in the control culture in a nonoxidizing medium. In the nonoxidizing medium, a dose-dependent effect was observed: at a higher concentration of juglone, there was a greater loss of ΔΨm, reaching up to 65% in cells treated with juglone at 10 μM, than that of 7.98% with juglone at 1 μM. When Xu et al. [29] evaluated the loss of ΔΨm in HL-60 cells; they determined that 8 μM juglone produced an effect after 1 h of treatment, which became statistically significant after 24 hours of treatment. These results were confirmed by Hall et al. [30] and Ji et al. [9], who indicated that an increase in the level of ROS would produce interference in the mitochondrial electron transport chain, meaning that its transmembrane potential would be

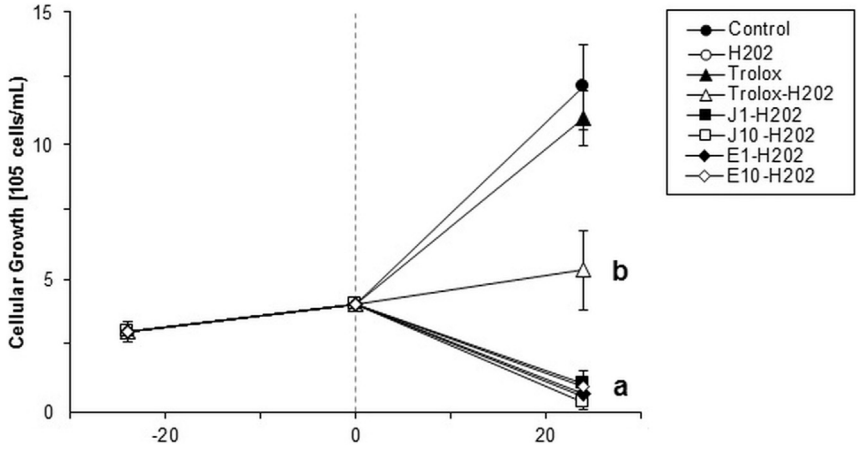

Figure 3. Effects of juglone-containing walnut green husk extract and purified juglone on the cell growth of HL-60 under oxidative conditions. Control: cultivation under optimum conditions; J1: culture with 1 μM purified juglone; J10: culture with 10 μM purified juglone; E1: culture with walnut green husk extract at 1 μM juglone concentration; E10: culture with walnut green husk extract at 10 μM juglone concentration. **a**:Significant differences when compared with Control; **b**: Significant differences when compared with Trolox

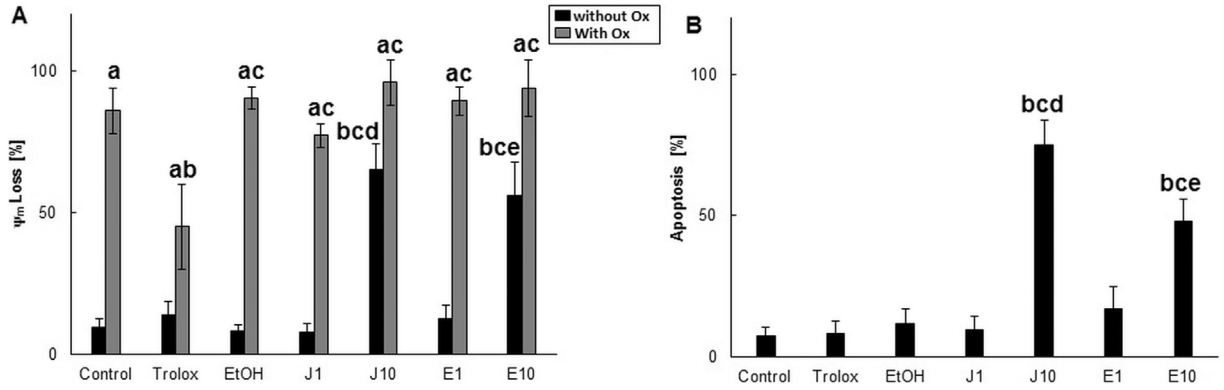

Figure 4. Evaluation of the effects of juglone and walnut green husk extracts containing juglone on A) the mitochondrial membrane potential (ΔΨm) of HL-60 under oxidative and nonoxidative conditions and on B) early apoptosis (sub-G1) of HL-60 cells under nonoxidative conditions. Control: cultivation under optimum growth conditions; Trolox: culture with the addition of 1 mM Trolox as a standard antioxidant; EtOH; culture with 0.14% v/v ethanol; J1: culture with 1 μM purified juglone (97%), J10: culture with 10 μM purified juglone, E1: culture with walnut green husk extract at 1 μM juglone concentration, E10: culture with walnut green husk extract at 10 μM juglone concentration. **a**: Significant differences when compared with counterpart culture with Ox; **b:** Significant differences when compared with Control; **c**: Significant differences when compared with Trolox; d: Significant differences when compared with J1; **e**: Significant differences when compared with E1.

affected by the high oxidation state and by mitochondrial DNA damage, among other factors. On the other hand, Montenegro et al. [24] reported that the integrity of the cell membrane of HL-60 cells was maintained intact after 24 h of incubation with juglone at 4 and 8 μM; however, some compounds derived from juglone, such as 5-methoxy-1,4-naphthoquinone, produced a loss of cell membrane integrity or depolarization (using rhodamine 123) at concentrations above 4 μM. No difference ($P > 0.05$) was observed when comparing the effects of pure juglone and the green husk extract containing 10 μM juglone, with results of 65% and 56.1% of ΔΨm lost, respectively. In an oxidizing culture medium, it was possible to observe no significant difference ($P > 0.05$) in the loss of mitochondrial membrane potential when the concentration of purified juglone or extract with juglone was increased, with a high loss of ΔΨm (over 80%) suggesting that the other compounds present in the extract did not produce a protective effect against oxidation in HL-60 cells. This result demonstrates the fact that antioxidants can act on different types of free radicals, and in this case, the compounds present in the GHL extract appear to act on peroxyl radicals given the high value of chemical antioxidant activity measured by ORAC [31]. However, these compounds do not have a strong effect on the scavenging of hydroxyl radicals such as those formed from H_2O_2 (a strong oxidant).

The effects of purified juglone and walnut green husk extract were also observed in the level of apoptosis of HL-60 cells. Apoptosis can be evaluated as the ability to arrest the cell cycle of the HL-60 line at the sub-G1 (G0/G1) phase [32]. As shown in Figure 4b, in the control culture, only 7% of cells were in the sub-G1 phase. In addition, it is possible to appreciate that in a nonoxidizing medium, the incorporation of Trolox or ethanol into the medium did not generate a significant increase in apoptosis; the same behavior was observed with 1 μM of purified juglone. However, a juglone concentration of 10 μM (either purified or in the extract) produced an increase of the cells in that state (sub-G1). This was also reported by Fang et al. [33] for SKOV3 ovarian cancer cells, in which 12.5 μM of pure juglone caused approximately 35% of cells to be in the sub-G1 (G0/G1) phase in comparison to 25% for the control culture without the addition of juglone, although the authors reported only 3% apoptosis when the analysis was performed using the annexin V method. In the case reported by Zhang et al. [6], when using stembark extracts of *J. mandshurica* on the hepatoma cell line Bel7402, between 43% and 58% of the cells were in the G0/G1 phase when the extract was used at concentrations of 120 to 30 μg/mL. On the other hand, in the annexin V method, Ji et al. [26] also reported a dose-dependent effect of juglone on the apoptosis of gastric cancer cells (SCG 7901), with up to 10% of apoptosis observed for 10 μM juglone compared to a control with only 0.17% apoptosis; the authors also reported this for MCF-7 breast cancer cells [9]. It is important to mention that the translocation of phosphatidylserine (detected by the annexin V method) occurs in early stages of apoptosis, whereas the entry into sub-G1 signifies advanced apoptosis; both methods have been reported for the determination of cellular apoptosis affected by different compounds, and dose-dependent effects were observed.

In this work, it was also possible to observe an important difference between the results obtained using pure juglone and those using the extract, i.e., 72% and 48% apoptosis, respectively ($P < 0.05$). This latter percentage may potentially be due to the less aggressive effect of the extract (which contains juglone).

Ji et al. [9] indicated that juglone could be transformed into a semi-quinone by means of an oxidation–reduction process; this semi-quinone would act on molecular oxygen, thereby converting it into a superoxide anion (O2-) and producing H_2O_2 enzymatically. These are ROS molecules and precursors of other ROS; hence, they would potentially generate oxidative damage in HL-60 cells, thus promoting cellular apoptosis.

4. Conclusions

The results conclude that walnut green husk extract possesses a higher antioxidant capacity (44,920 ORAC units) than some berries and equivalent to basil and cacao. The walnut green husk extract expressed an antiproliferative effect in HL-60 cells when juglone concentrations in the extract were 1 μM and 10 μM. The extract with 1 μM juglone did not affect the viability of normal cells, while at higher concentrations (10 μM), there is loss of viability of both cancerous and healthy cells.

Under the conditions studied, there are two proposed mechanisms underlying the effect of walnut green husk extract over the viability of HL-60 cells: first, a pro-oxidant effect of juglone produces a loss of mitochondrial membrane potential in nonoxidizing media and leads to a lack of protection by other nonjuglone compounds in oxidizing media, supporting the results of Ji et al. [8], and second, the induction of the early stages of apoptosis (sub-G1 phase), supporting the results of Zhang et al. [6]. Despite the results, more studies should be conducted to elucidate the mechanism underlying the action of juglone extract on HL60 cell viability.

The effect of juglone extract on HL-60 cells shows the advantage of using a natural extract in the treatment of diseases such as cancer without the need to perform complex processes to purify bioactive compounds.

The information generated adds value to an agro-industrial discard rich in juglone and, at the same time, contributes to the theoretical basis that could guide the development of new drugs or nutraceuticals and the clinical application of natural extracts from plant sources.

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Declarations of Interest

None.

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