Artículo original

Efecto antiproliferativo y citotóxico del extracto de *Ginkgo biloba* L. en una línea de cáncer cervical

Antiproliferative and cytotoxic effect of *Ginkgo biloba* L. extract in a line of cervical cancer

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RESUMEN

Introducción: El empleo del extracto de *Ginkgo biloba* L. como agente contra el cáncer es controversial. Si bien su consumo disminuye riesgo de cáncer de ovario, sin evidencia clara en el tratamiento de otros cánceres ginecológicos (cuello uterino).

Objetivo: Evaluar el efecto del extracto estandarizado de *Ginkgo biloba* L. sobre la proliferación y supervivencia en líneas celulares de cáncer de cuello uterino y de células embrionarias de riñón humano inmortalizadas.

Métodos: Se evaluó el extracto de *Ginkgo biloba* (0-10000 µg/mL) en la proliferación y supervivencia de líneas celulares de cáncer de cuello uterino y de riñón, determinando viabilidad celular mediante ensayo de proliferación celular a 24-48 h de tratamiento.

Resultados: La concentración de 1000 µg/mL, redujo significativamente (p < 0,001) la proliferación de ambas líneas celulares, medida como concentración inhibitoria 50 (células de cuello uterino = 1,137 ± 251,5 µg/mL; células de riñón = 917,6 ± 220,8 µg/mL) y aumentó 3,7 veces tiempo de duplicación de células de cuello uterino, pero solo 1,9 veces, las de células de riñón. El ensayo de citotoxicidad mostró que concentraciones superiores a 2,500 µg/mL redujeron significativamente viabilidad celular a las 24 hr (células de cuello uterino = 1,962 ± 368,8 µg/mL; células de riñón= 2,335 ± 185,0 µg/mL).

Conclusiones: Aunque los resultados muestran las propiedades antiproliferativas y citotóxicas del extracto, la concentración requerida es muy alta. Por tanto, su uso terapéutico en mujeres con cáncer de cuello uterino debe considerarse con precaución.

Palabras clave: líneas celulares de cáncer cervical; actividad citotóxica; actividad antiproliferativa; *Ginkgo biloba.*

ABSTRACT

Introduction: The use of *Ginkgo biloba* L. extract as an anti-cancer agent is controversial. Although its consumption decreases the risk of ovarian cancer, there is no clear evidence in the treatment of other gynecological (cervical) cancers.

Objective: To evaluate the effect of standardized extract of *Ginkgo biloba* L. on proliferation and survival in cervical cancer cell lines and immortalized human kidney embryonic cells.

Methods: *Ginkgo biloba* extract (0-10000 µg/mL) was evaluated in the proliferation and survival of cervical and kidney cancer cell lines, determining cell viability by cell proliferation trial at 24-48 h of treatment.

Results: The concentration of 1000 µg/mL significantly reduced (p < 0.001) the proliferation of both cell lines, measured as inhibitory concentration 50 (cervical cells = 1.137 ± 251.5 µg/mL; kidney cells = 917.6 ± 220.8 µg/mL) and increased 3.7 times the doubling time of cervical cells, but only 1.9 times, those of kidney

cells. The cytotoxicity trial showed that concentrations above 2,500 µg/mL significantly reduced cell viability at 24 hr (cervical cells = 1,962 ± 368.8 µg/mL; kidney cells = 2,335 ± 185.0 µg/mL).

Conclusions: Although the results show the antiproliferative and cytotoxic properties of the extract, the required concentration is very high. Therefore, its therapeutic use in women with cervical cancer should be considered with caution.

Keywords: cervical cancer cell lines; cytotoxic activity; antiproliferative activity; *Ginkgo biloba*.

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Introduction

Ginkgo biloba extract is used worldwide as a food supplement that promotes general health status. However, evidence of its genotoxic and carcinogenic effects has raised several doubts about its routine use safety.[1] In addition, while the regular consumption of *Ginkgo biloba* preparations seems to decrease ovarian cancer risk, and the crude extract and *Ginkgo* secondary metabolites reduced serous ovarian cancer cells' proliferation,[2,3] the effect on other malignant gynecological neoplasms, like cervical cancer, remains little explored.

Cervical cancer is the fourth most frequent neoplasia in women and the second cause of death after breast and lung cancer.[4,5] Early stages of cervical cancer are well treated; in contrast, late stages and metastatic cancer have a poor prognosis.[6] The human papillomavirus (HPV) is the principal cause of cervical cancer;[7] however, HPV vaccination and early detection campaigns have reduced this illness's incidence and mortality. Nevertheless, despite the current treatment strategies, it still is a significant public health problem.[7]

The standardized extract of *Ginkgo biloba* (EGb 761, trade name) is a mixture consisting mainly of 24 % flavonoids (quercetin, kaempferol, and isorhamnetin), 6 % terpene lactones (3.1 % ginkgolides and 2.9 % bilobalides), and 5-10 % organic

acids,[8] with antioxidant, anti-proliferative, and apoptotic activity reported in different cell lines.[9,10,11,12] Also, it has been evaluated in animal models of diseases related to oxidative stress and inflammation[13,14] and human patients with pancreas, colon, stomach, and liver cancer.[15,16,17,18]

Moreover, some studies have documented the anti-proliferative and pro-apoptotic properties of flavonoids from EGb761[2,19,20] and ginkgolide B[21] in cervical cancer cell lines. However, the effect of the commercial leave extract on cervical cancer cell lines remains unknown. Consequently, the present study aimed to evaluate the standardized EGb 761 on the proliferation and survival of cervical cancer (He La) and immortalized Human Embryonic Kidney (HEK 293) cell lines. The results obtained in this study demonstrate that EGb 761 has a similar anti-proliferative and cytotoxic effect on He La and HEK 293 at high concentrations.

Methods

Reagentes

EGb 761 (Vasodil®) is a commercial natural extract obtained from dried green leaves of the *Ginkgo biloba L.* tree (Author Linnaeus, Carl von; Family Ginkgoaceae Engl.) and manufactured in Mexico under the license of Dr. William Schwabe by Nycomed S.A. de C.V. There is 40 mg of a dry extract standardized to 9.6 mg of flavonoid glycosides per mL of solution, calculated as quercetin and kaempferol. RPMI-1640 medium, Sodium pyruvate, Penicillin-streptomycin solution, Phosphate buffer solution, Trypsin-EDTA (ethylenediaminetetraacetic acid) solution, and MTT [3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium bicarbonate and DMSO (dimethyl sulfoxide) were obtained from Sigma Chem (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biowest (Nuailé, France).

Cell Culture

Mexico's National Institute of Cancer donated the cell lines that were authenticated as He La and HEK 293 cells. He La is an epithelial cell isolated from a cervix adenocarcinoma lesion and contain human papillomavirus. HEK 293 is a cell line originally isolated from a human embryonic kidney. Both cell lines were cultured in RPMI medium supplemented with 8 % SFB, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (P/S) and were incubated at 37°C in a 5 % carbon dioxide (CO_2) atmosphere. Cultures were visually monitored during the treatment.

Cell proliferation assay

Both, He La and HEK 293 cell lines were cultured in 96-well multiplates (0.32 cm^2 culture surface) at 1.5 x 10^4 cells/ cm^2. At the beginning of the exponential growth phase, cultures were incubated with different concentrations of EGb 761 (ten-fold serial dilutions from 10000 to 0.1 µg/ mL). Cultures without EGb 761 were used as control. The MTT assay determined proliferation at 24 and 48 hr. This assay is based on the metabolization of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt in insoluble formazan crystals in the cytoplasm of viable cells. Briefly, the medium was replaced by 50 µL of MTT (5 mg/ mL) and incubated for four hours. Subsequently, 200 µL of DMSO was added to dissolve the formazan crystals. The absorbance was determined at 570 nm using a microplate reader (Stat Fax 4200, Awareness Technology). Three independent experiments were performed in triplicate.

Cytotoxic activity

To determine the cytotoxic capacity of EGb 761, He La and HEK 293 cell lines were cultured in 96-well multiplates (0.32 cm^2 culture surface) until reaching 100 % confluence; replaced the media with the different treatments of EGb 761 (100, 1000, 2500, and 5000 µg/ mL). Cultures without EGb 761 were used as control. The MTT assay measured cell viability, determining the viability percentage at 24 and 48 hr of treatment, respectively. Three independent experiments were performed in triplicate.

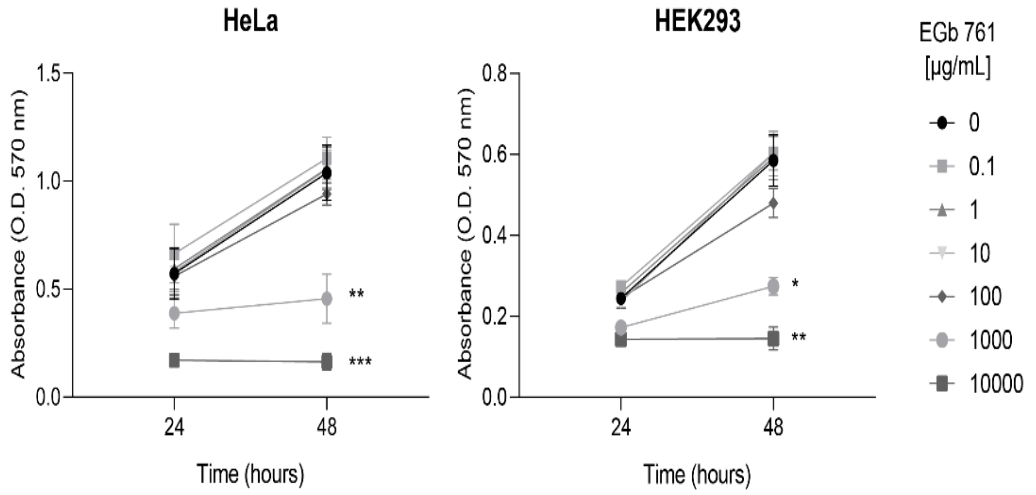
Statistical analysis

The R program (version 3.6.1) performed the Statistical analysis. The effect of extract concentration and treatment time on cell lines was evaluated by the bifactorial ANOVA test in proliferation and cytotoxicity assays, followed by post-hoc analysis using simple contrasts with adjustment of *p*-value by the Sidak method. In cases where the model's residuals did not meet the assumptions of normality, homoscedasticity, or independence, evaluated data through a robust two-way ANOVA with the WRS2 package. Both assays' inhibitory concentration (IC_{50}) values were obtained using the non-linear regression model of dose-response curves by the equation log (inhibitor) vs. normalized response - variable slope. Graphs and dose-response curves were performed in GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

Results

Anti-proliferative activity of EGb761

The effect of EGb 761 on proliferation was evaluated on He La and HEK 293 cells treated with increasing concentrations of EGb 761 for 24 and 48 hr. The MTT assay revealed a decrease in cell density after 24 hr with 1000 µg/ mL, which becomes more evident after 48 h, while the concentration of 10000 µg/ mL was markedly cytotoxic (fig. 1). These results were corroborated by daily cell microscopic observation (fig. 2). To verify the anti-proliferative effect of EGb 761, the doubling time of cell population, which is characteristic in each cell line, was determined. Interestingly, the 1000 µg/ mL concentration increased 1.9-fold the doubling time of cell population in the HEK 293 line comparing to the control, while on the He La cell line, the increase in doubling time was much more marked (3.7-fold). Although both cell lines were affected, He La cells take longer (102.8 hr) to double in size than HEK cells (35.76 hr). This data suggested that EGb 761 delays cancer cell growth than in control cells. The comparison of IC_{50} did not show significant differences between the corresponding values of each cell line (HEK 293 IC_{50}= 1137 ± 251.5 µg/ mL; He La IC_{50} = 917.6 ± 220.8 µg/ mL, p = 0.5383) (fig. 3).



Analysis of cell proliferation by the MTT assay in He La and HEK 293 cells incubated with different concentrations of EGb 761 for 24 and 48 hr. Dots represent the mean ± sem of absorbance.
Asterisks indicate significant differences with respect to the control (**p < 0.01, ***p <0.001).

Fig 1 - EGb 761 extract decreases the proliferation of cell lines.

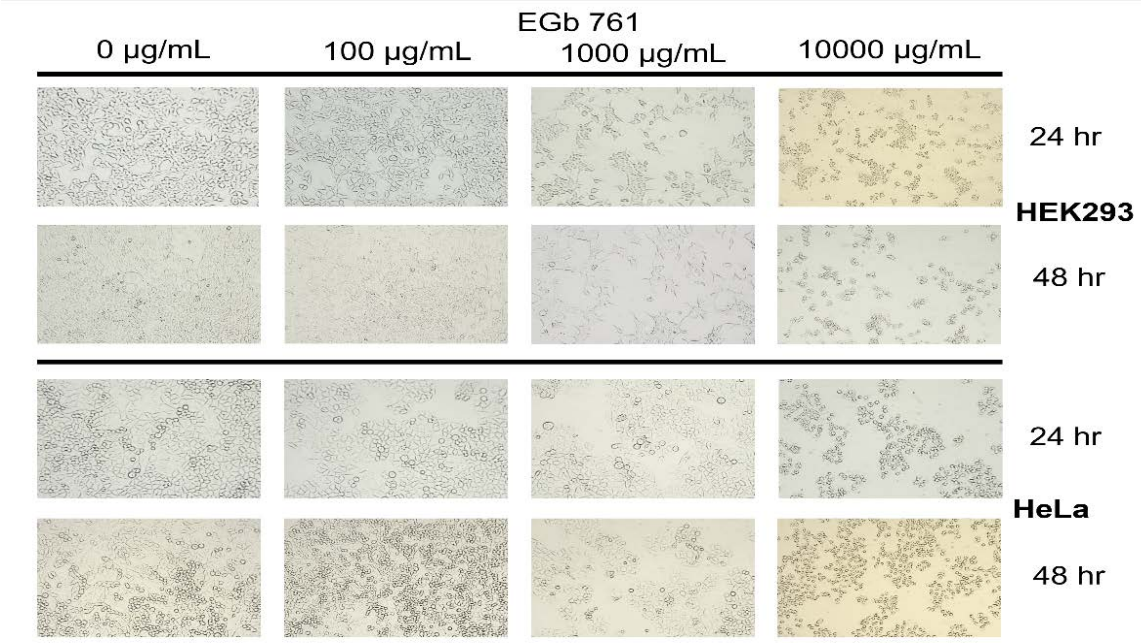
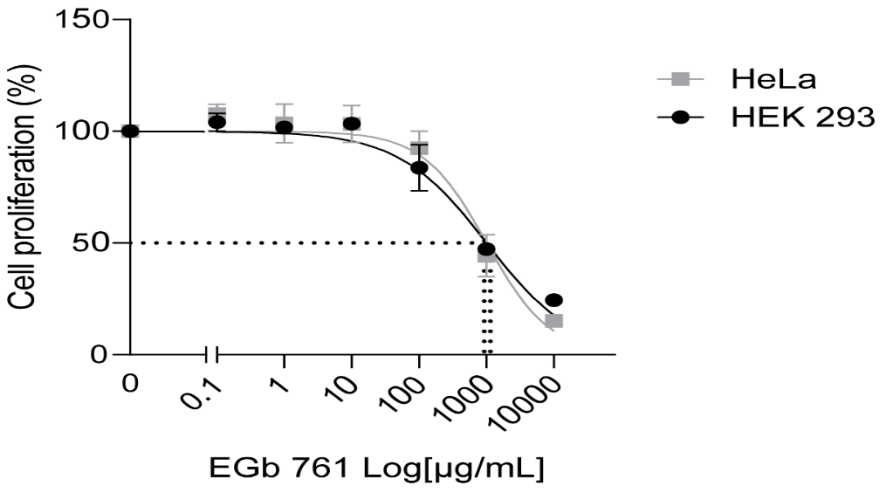


Fig 2- Proliferation assay microphotographs of He La and HEK 293 (10X) cell lines show the cultures' appearance from 0 to 10000 µg/ mL of EGb 761 at 24 and 48 hr incubation.

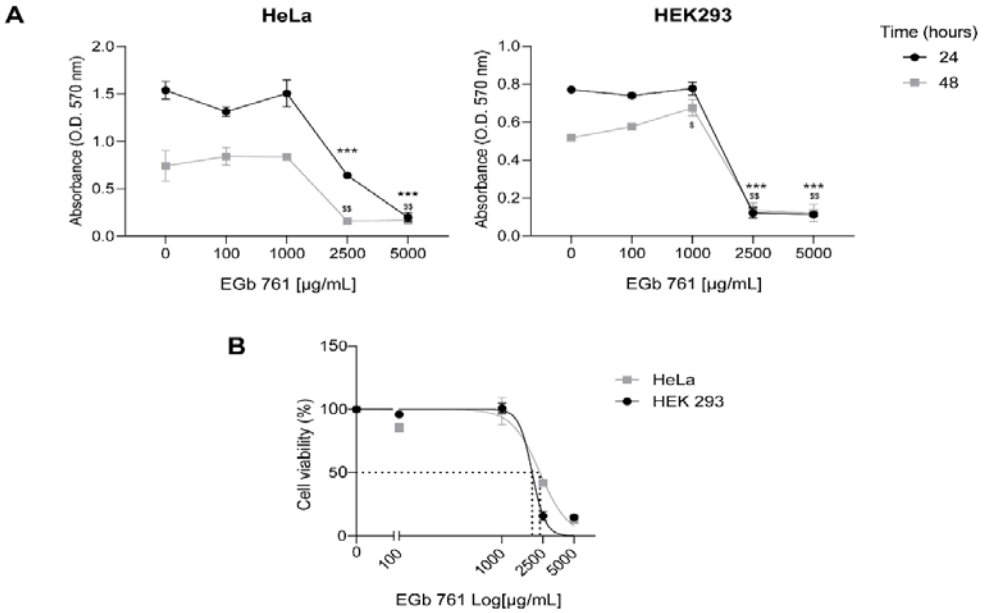


Dose-response curve of the He La and HEK 293 cell lines treated for 48 hr with the EGb 761 extract. The graph shows the estimated IC50 value of proliferation for both cell lines (dot lines). Dots represent the mean ± sem of the percentage of cell proliferation.

Fig 3 - Determination of the anti-proliferative activity of EGb 761.

Cytotoxic activity of EGb 761

A cytotoxicity assay was carried out on both cell lines' confluent cultures to corroborate that the inhibition of proliferation observed is not due to a cytotoxic effect of EGb 761. Viability results from the MTT assay demonstrated that EGb 761 is not cytotoxic for both cell lines within the range of 100 to 1000 µg/mL, but significantly affects the survival of both cell lines at concentrations greater than or equal to 2500 µg/ mL (fig. 4A). Also, at 48 hr, control cultures of both cell lines showed a decrease in absorbance, possibly due to the deterioration of the conditions of a confluent culture. Interestingly, the absorbance observed in the HEK 293 cell lines incubated with 1000 µg/mL of EGb at 48 hr was higher than in with control cells, with significant differences (p = 0.0338). In contrast, this increase was not observed in the He La cell line (fig. 4A). The comparison of the dose-response curves for 24 hr suggests a differential effect on both cell lines, with the HEK 293 line being more sensitive to its cytotoxic action; however, we did not find significant differences between the IC_{50} values (p = 0.8004) (HEK 293 IC_{50} = 1,962 ± 368.8 µg/ mL; He La IC_{50}=2,335 ± 185.0 µg/ mL; mean ± sem) (fig. 4B). These results were corroborated by daily cell microscopic observation (fig. 5).



(A) Cytotoxicity analysis of EGb 761 extract by MTT assay at 24 and 48 h. Dots represent the mean ± sem. Symbols indicate significant differences between concentration treatment and its respective control (* for 24 / 48 h; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(B) Dose-response curve of the cytotoxicity assay in He La and HEK 293 cell lines treated for 24 h with EGb 761. The graph shows the estimated IC_{50} value for both cell lines (dot lines). Dots represent the mean ± sem.

Fig 4 - EGb761 extract decreases the viability of He La and HEK 293 cell lines at high concentrations:

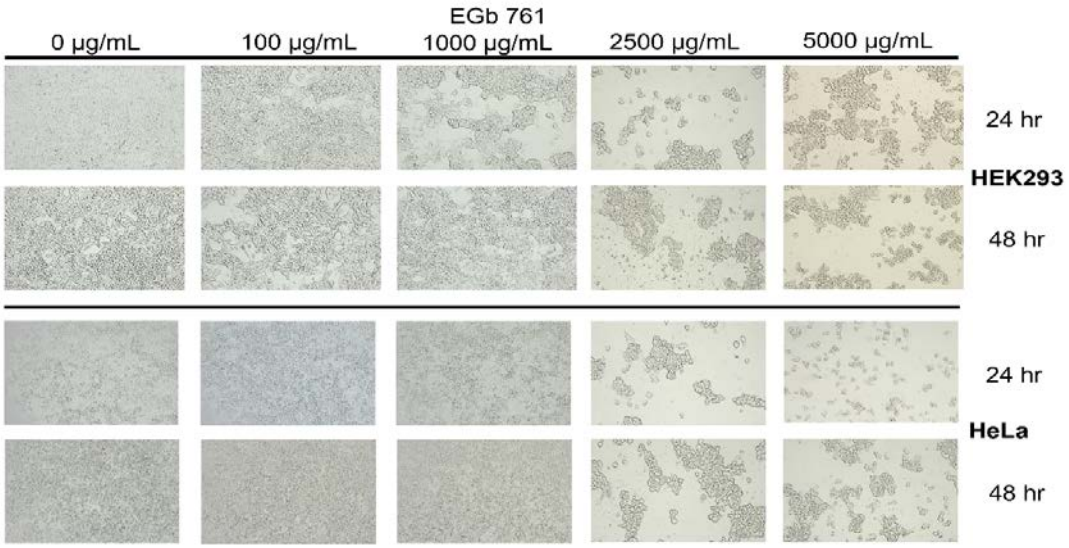


Fig 5 - Cytotoxicity assay microphotographs of He La and HEK 293 (10X) cell lines show the cultures' appearance from 0 to 5000 µg/mL of EGb 761 at 24 and 48 h incubation.

Discussion

EGb 761 has been reported as a chemo and radiotherapy sensitizing agent against pancreatic,[16] colorectal,[17] gastric,[18] and lung cancer.[22] This sensitizing effect is associated with its pro-apoptotic, anti-proliferative, and cytotoxic capacity, widely reported in both cell and animal models.[10,12,23,24,25,26,27] Moreover, *Ginkgo biloba* preparations seem to reduce ovarian cancer risk;[3] however, the effectivity of EGb 761 on cervical cancer has not been reported, despite the effect of secondary metabolites in different cervical cancer cell lines.[2,19,20,21] We probed the anti-proliferative and cytotoxic activity of EGb 761 in two different cell lines, one derived from cervical cancer (He La) and the other from the embryonic origin (HEK 293). EGb 761 significantly inhibits the proliferation of both cell lines at equivalent concentrations. However, the doubling time was longer in the He La (3.7-fold) than in the HEK 293 line (1.9-fold), suggesting differences in the magnitude of the anti-proliferative effect on these lines, with a marked delay in He La cells growth.

The anti-proliferative activity of EGb 761 has been previously reported in different cancer models of breast[12] and liver cancer cell lines,[9,28] but with different sensitivities. For example, Czauderna's group[28] showed an anti-proliferative effect of EGb 761 with IC_{50} ranges between 150 to 480 µg/ mL in three different liver cancer cell lines. However, Chao and Chu[9] indicated the same effect on Hep G2 and Hep 3B liver cancer lines, but at higher concentrations, with 50 and 45 % inhibition rates for 750 µg/ mL and from 48 and 39 % for 1000 µg/ mL, respectively. Our results agree with this, with IC_{50} close to 1000 µg/ mL. The differences in the range of the anti-proliferative effects reported would be related to the sensitivity of the type of cancer studied. Also, the increase in the doubling time observed in both lines could be related to the cell cycle, since in colon and gastric cancer cells, the accumulation in G0/ G1 phases was observed when cells were exposed to different concentrations of EGb 761.[10,29] It is essential to consider that, although the HEK 293 cell line has been employed as a standard cell control in several studies that aimed to probe the anti-cancer properties of drugs and phytochemicals,[30,31,32] the unstable karyotype could increase its

tumorigenic potential, so it is used like an *in vitro* tumor model since transformation occurs at higher passages.[33,34]

The results of the cytotoxicity assay demonstrate that EGb 761 does not affect cell viability at concentrations where it exerts its anti-proliferative effect (1000 µg/ mL) and only does so at concentrations greater than 1000 µg/ mL (HEK 293 IC_{50}= 1,962 ± 368.8 µg/ mL; He La IC_{50}= 2,335 ± 185.0 µg/ mL) after 24 hr. The effect of EGb 761 has not been reported on cervical cancer cell lines; nevertheless, in a study conducted by Xu et al.,[35] they showed that biosynthetic silver nanoparticles with aqueous extracts of *Ginkgo biloba* leaves (GB-AgNPs) decrease cell viability on He La and Si Ha cell lines. However, Stark and Behl[36] reported that EGb 761 did not decrease the viability of HEK 293 even at 300 µg/ mL.

Besides, there is evidence of the cytotoxic effect of EGb 761 on breast, skin, colon, and liver cancer cell lines,[9,10,26,37] with a broad spectrum of effective concentrations ranging from 100 to 1000 µg/ mL. Thus, for example, on breast carcinoma cell line over-expressing estrogen and progesterone receptors (MCF-7) the cytotoxic activity is significant at 1000 µg/mL after 72 hr of incubation.[27] However, this disagrees with the high cytotoxic concentrations of EGb 761 showed in this study.

The microscopic observation of both cell lines' cultures showed no apparent signs of cell damage up to 1000 µg/ mL, suggesting that the anti-proliferative effect of EGb 761 is not mediated by necrotic death. A pro-apoptotic mechanism of the EGb 761 extract is also possible, as reported on breast, liver, skin, and colon cancer cell lines,[10,26,28,37] therefore, it would be essential to determine if EGb 761 regulates pro-apoptotic proteins' expression in these He La and HEK 293 cell lines. In this sense, *Xu* et al.,[35] suggested a possible mitochondrial pathway, mediated by an increase in the release of cytochrome C and the cleavage of the 9 and 3 caspases.

On the other hand, we found that the EGb 761 extract improved the survival of HEK 293 confluent cultures. Interestingly, *Xu* et al.,[35] also showed that GB-AgNPs slightly increased the viability of He La and Si Ha lines at low concentrations. However, in our study, this difference occurs only in the HEK 293

cell line and not in the He La cell line, suggesting differences in action mechanisms against oxidative stress. More experiments are required to evaluate this possibility.

It is essential to consider that the antioxidant effect of EGb 761[13,28] could stimulate rather than halting cancer development[1] as reported for other antioxidant agents used as food supplements depending on the degree of neoplasm progression[38] or the used dose of *Ginkgo biloba*.[39] Furthermore, the therapeutic use of EGb 761 in cancer is limited by conflicting evidence reported in clinical cases studies about the risk of developing different types of cancer, i.e., breast, and colon was increased, including several grades of morphological abnormalities related to liver, thyroid, and nose cancer in experimental models due to large consumption doses and the presence of some mutagenic metabolites like ginkgotoxin.[1]

Altogether, our results demonstrate that standardized EGb 761 extracts have a similar anti-proliferative and cytotoxic effect on cervical cancer and embryonic cells. While this finding could support the anti-cancer properties of EGb 761, it is crucial to consider that the concentrations at which it exerts these effects are very high and could also be cytotoxic for normal cells; for that reason, its therapeutic use in patients with cervical cancer should be considered with caution. More studies are required to evaluate the safety of the therapeutic use of EGb 761 in cervical cancer treatment.

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Conflict of interest

The authors declare no conflict of interest.

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