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Article

Comparative Cytotoxic Study of Wild Plant Extracts, Their Callus Cultures and 2,3-Dihydrobenzofuran from *Ageratina pichinchensis*

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Abstract: The cytotoxic effect of four extracts obtained from the wild plant and two from a culture of *Ageratina pichinchensis* calluses on cell lines: prostate carcinoma, cervical cancer, hepatocellular carcinoma, human hepatoma, lung cancer and cellular keratinocytes was evaluated; the extracts were obtained with ethyl acetate and methanol, leaves and stems were used from the wild plant. The results of the cytotoxic evaluation showed that only the ethyl acetate extract obtained from the callus culture showed an effect on the cervical cancer cell line (HeLa) with an IC_{50} of $94.79 \pm 2.0 \mu\text{g/mL}$. The purification of this extract allowed obtaining a benzofuran type compound as the main product, which was identified as 2,3-dihydrobenzofuran. The cytotoxic evaluation of this compound showed an important effect against the HeLa cell line with an IC_{50} of $23.86 \pm 2.5 \mu\text{g/mL}$. These results contribute to the development of alternatives for the production of compounds for the treatment of cancer.

Keywords: cytotoxic activity; 2,3-dihydrobenzofuran; cervical cancer

1. Introduction

Ageratina pichinchensis is a plants species endemic to the state of Morelos popularly known as "water sheet" and "axihuitl" mainly, it grows in the municipalities of Amatlán and Tepoztlán, where it is used for the treatment of gastric ulcers, healing, and diseases related to inflammatory events [1], it should be noted scientific phytochemical and pharmacological studies have validated its ethnomedical use [2-7], likewise, the establishment of its cultures of calluses and cells in suspension with the capacity to biosynthesize compounds structurally similar to those produced by the wild plant, which showed an important anti-inflammatory effect, has been reported. It should be noted that the compounds 3-epilupeol and a benzofuran were identified that had not been reported in the wild plant, whose production was improved in an airlift reactor 1.82 and 1.35 folds, respectively [8-10].

However, other biological properties have not been explored, such as those related to the cytotoxic effect, which has become relevant due to the impact in the health area in the search for

molecules for the treatment of neoplastic diseases. The WHO estimates that cancer is the leading cause of death in the world and more than 18 million deaths were reported worldwide in 2022 alone, the most common being breast, lung, colon, rectum, and prostate [11, 12], this has led to the search for and development of solutions from different approaches, given that the causes are diverse and diagnosis is also a challenge, which is why multiple research groups around the world have dedicated themselves to the task of searching for therapeutic compounds of synthetic and natural origin [13-15] to be applied in therapies or treatments that are already applied in order to improve their effectiveness; one of the common treatments in cancer patients are chemotherapies, which make use of molecules that act at different levels of the cell cycle, successfully saving lives or improving the quality of patients, despite the percentage of efficacy of chemotherapies, the search for therapeutic agents continues to be a necessary due to the high global demand [16-18].

In view of the above, medicinal plants are a prominent source of compounds with cytotoxic effects in different types of cancer, some of the molecules commonly used in chemotherapies are the tetracyclic diterpenoid compound taxol, which is isolated from the bark of species of the genus *Taxus*, and is commercially known as paclitaxel® and it has been impossible to develop the synthetic route due to the complexity of the molecule, which has led to its direct obtaining from the plant, with the disadvantage that trees require 15 years of age to be able to biosynthesize the compound [19,20]; on the other hand, the vinblastine and vincristine alkaloid-type compounds from *Catharanthus roseus* present yields below 0.001% and their cost ranges from one billion dollars per kilogram, but they are used for their efficacy in chemotherapies for patients with leukemia [21]; other potentially anticancer alkaloids are colchicine, vindesine, vinorelbine, podophyllotoxin, decotaxel, campotecin, curcumin, apigenin and vincamine, all of which have a common factor in their origin from plant species [22-26]. On the other hand, extracts of medicinal plants have also shown a cytotoxic effect where the compounds participate synergistically, as has occurred with the species *Aristolochia baetica*, *Artemisia annua*, *Fagonia indica*, among others [27,28]. However, there are numerous plant species around the world that have not yet been studied in Mexico alone, more than 300 endemic plants of different families and genera are used, of which only a proportion have been scientifically studied [29,30]. On the other hand, there are reports of cultures of calluses and cells in suspension of plant species whose main advantage is the production of bioactive compounds in a constant and controlled manner, which is a useful alternative because medicinal plants in wild conditions are affected by different seasonal, environmental, geographical conditions, etc. These factors are determinant in the production and yields of secondary metabolites and put medicinal plants at a disadvantage as a sufficient source for world needs in tumor pathologies. For this reason, this paper reports the study of wild plant extracts and callus cultures of the species *A. pichinchensis* whose scientific findings demonstrate its cytotoxic effect on the HeLa cell line, associating the chemical constituents with the activity observed in the extracts.

2. Results and Discussion

2.1. Cytotoxic evaluation of extracts

The cytotoxic activity of ethyl acetate and methanol extracts from callus cultures and wild Plants of *A. pichinchensis* was evaluated at different concentrations on cell lines: human prostate carcinoma (PC-3), cervical cancer (HeLa), hepatocellular carcinoma (Huh-7), human hepatoma (HepG2), breast tumor cells (MCF7), and keratinocyte cells (Hacat). The methanolic and ethyl acetate extracts of the wild plants and their callus cultures did not show a significant effect on the cell lines, while the ethyl acetate extract of the wild plant leaves had a slight effect on the HeLa cells and PC-3; however, the ethyl acetate extract of the callus cultures showed a greater effect on the cell lines (Table 1), with the HeLa cell line where this effect was more relevant.

Table 1. Cytotoxic activity of ethyl acetate and methanol extracts from callus cultures and of wild plants of *A. pichinchensis*.

Cellular Line	Wild Plant Leaves		Wild Plant Stems		Callus Cultures		Paclitaxel (nM)*
	EAE (µg/mL)	ME (µg/mL)	EAE (µg/mL)	ME (µg/mL)	EAE (µg/mL)	ME (µg/mL)	
HeLa	161±4.9	>200	>200	>200	94.79±2.0	150.9±7.5	20±1.2

PC-3	188.6±10.1	>200	>200	>200	121.2±9.2	168.6±4.5	15±2
Huh-7	>200	>200	>200	>200	132.8±8.5	>200	25±3
HepG2	>200	>200	>200	>200	122.9±2.5	>200	10±1.5
MCF-7	>200	>200	>200	>200	191.3±6.4	>200	22±4
Hacat	>200	>200	>200	>200	>200	>200	114.7±15

EAE = Ethyl acetate extract; ME = methanol extract. * Positive control.

A. pichinchensis, is widely used in traditional Mexican medicine. It is used to treat diseases caused by fungal and skin infections, wounds, as well as to relieve pain and treat gastric ulcers, and anti-inflammatory effects have been reported. Extracts from this plant have shown antifungal activity against *Trichophyton mentagrophytes*, *T. rubrum* and *Candida albicans*, and have shown therapeutic effectiveness in patients with vulvovaginal candidiasis [7, 31].

It should be noted that the ethyl acetate extract of the callus culture contains compounds (1-11) (Fig.1), with compound 7 (2,3-dihydrobenzofuran) being a major one.

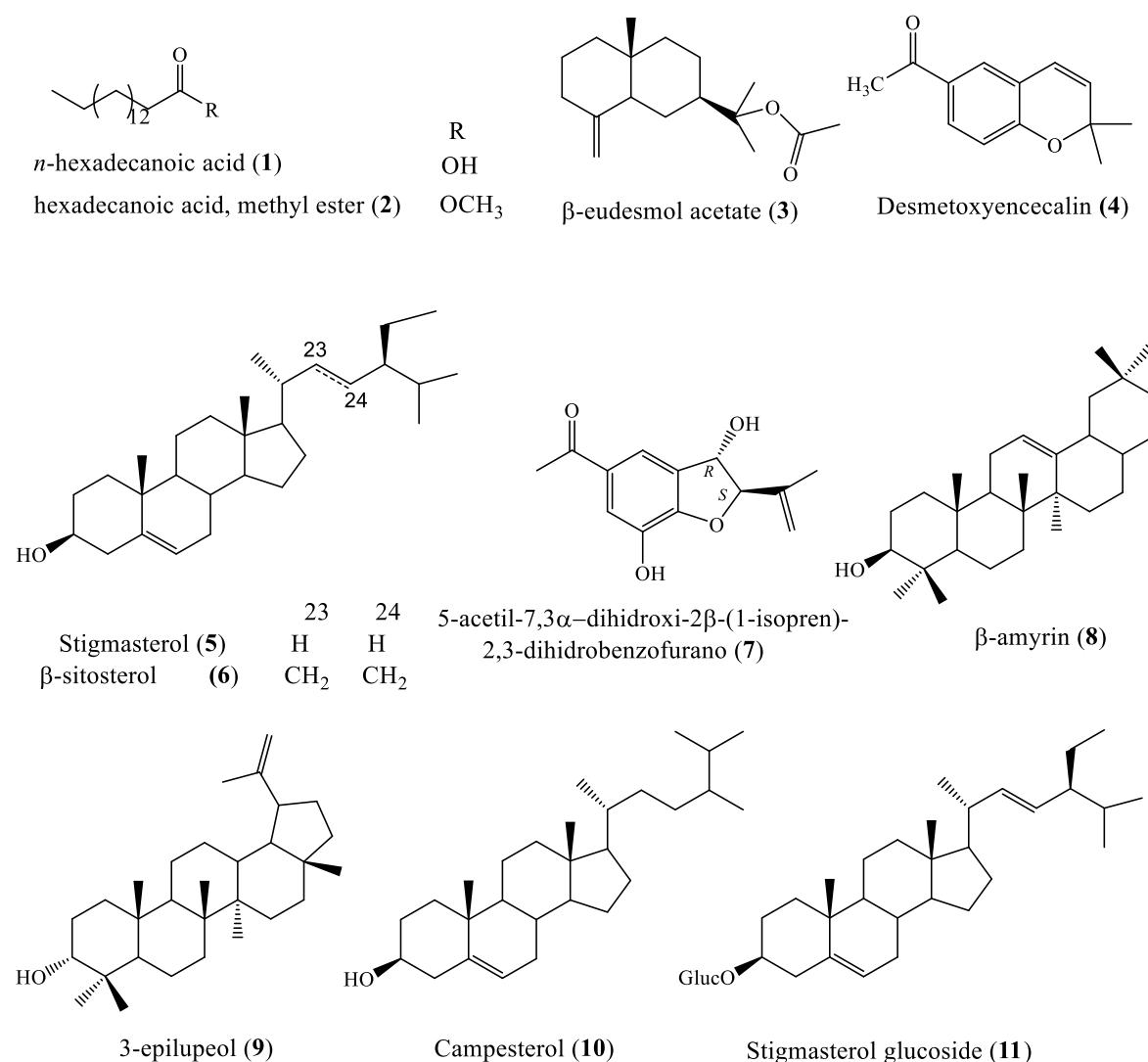


Figure 1. Compounds identified in the ethyl acetate extract of *A. pichinchensis* callus.

The compound campesterol (10) has been reported as an important phagocytosis suppressor and inhibitor of lipopolysaccharide in RAW 264 macrophage cells [32], in addition, extracts of different plant species with anti-inflammatory effect have revealed that the presence of the compound participates in the biological effect attributed to the extracts, such as in *Cajanus cajan* L. seeds, *Ananas comosus* leaves, *Allium schoenoprasum* L. leaves and in *Opuntia ficus-indica* seed oil [33-36] among other species.

Another compound identified in the extract is hexadecenoic acid (**1**), which has been shown to be an important inhibitor of the PLA2 enzyme that plays a role in inflammation of blood vessels and favors the development of atherosclerosis [37], it should be noted that it has been reported in other species, which stand out for their anti-inflammatory effect evaluated at the extract level, such as the species *Chasmanthe aethiopica*, *Zostera japonica* and *Jatropha curcas* [38-40]; likewise, the pentacyclic triterpene-type compound β -amyrin (**8**) has also exhibited anti-inflammatory effect induced in lipopolysaccharide and interferon- γ and even significantly reduces the expression of the levels of proinflammatory factors TNF- α , IL-1 β , IL-6, PGE-2 and COX-2 [41,42], in addition, its cytotoxic effect was reported in the MCF-7 breast cancer cell line with IC₅₀ 15.5 μ g/mL, and it has also shown a cytotoxic effect in colon, ovarian, cervical, lung, and breast cancer lines [43-47], and it has been reported in species from different families such as *Alstonia boonei*, *Protium heptaphyllum* and *Gymnosporia montana* [48-51] in addition, it has been attributed an antioxidant effect, as it has been in the species *Myrcianthes pungens* and *Celastrus hindsii* [52,53]; on the other hand, the compound 3-epilupeol (**9**) has revealed an important anti-inflammatory effect and the species of the genus *Bursera* are the main ones in producing it, whose extracts at the extract level reveal cytotoxic activity in most cell lines [54-56]; on the other hand, the stigmasterol glycoside compound has revealed a cytotoxic effect on kidney, breast and liver tumor cells [57-60], other reports argue its anti-inflammatory and antimicrobial effect antimicrobial [61-63]; finally, benzofuran compound has been reported as anti-inflammatory [10].

These scientific reports show the therapeutic effect attributed to the compounds and their respective plant species, therefore, the cytotoxic effect identified in the ethyl acetate extract of callus cultures is due to the presence of the compounds that act synergistically, significantly enhancing their effect, outstandingly, the extract biosynthesizes the compounds in a particular and constant way, which represents an advantage over wild plants whose production of compounds depends on environmental, geographical, and seasonal factors, among others, which is why calluses represent an important source of molecules with therapeutic effects as supported by numerous scientific reports.

Several studies indicate antiviral, antioxidant, antimicrobial, antitumor activities of dihydrofurans [64-66]. Considering this analyzed the effect of 2,3-dihydrobenzofuran on the inhibition of cell proliferation of the study cell lines. A clear effect on inhibition of HeLa cells is observed in **Figure 2**.

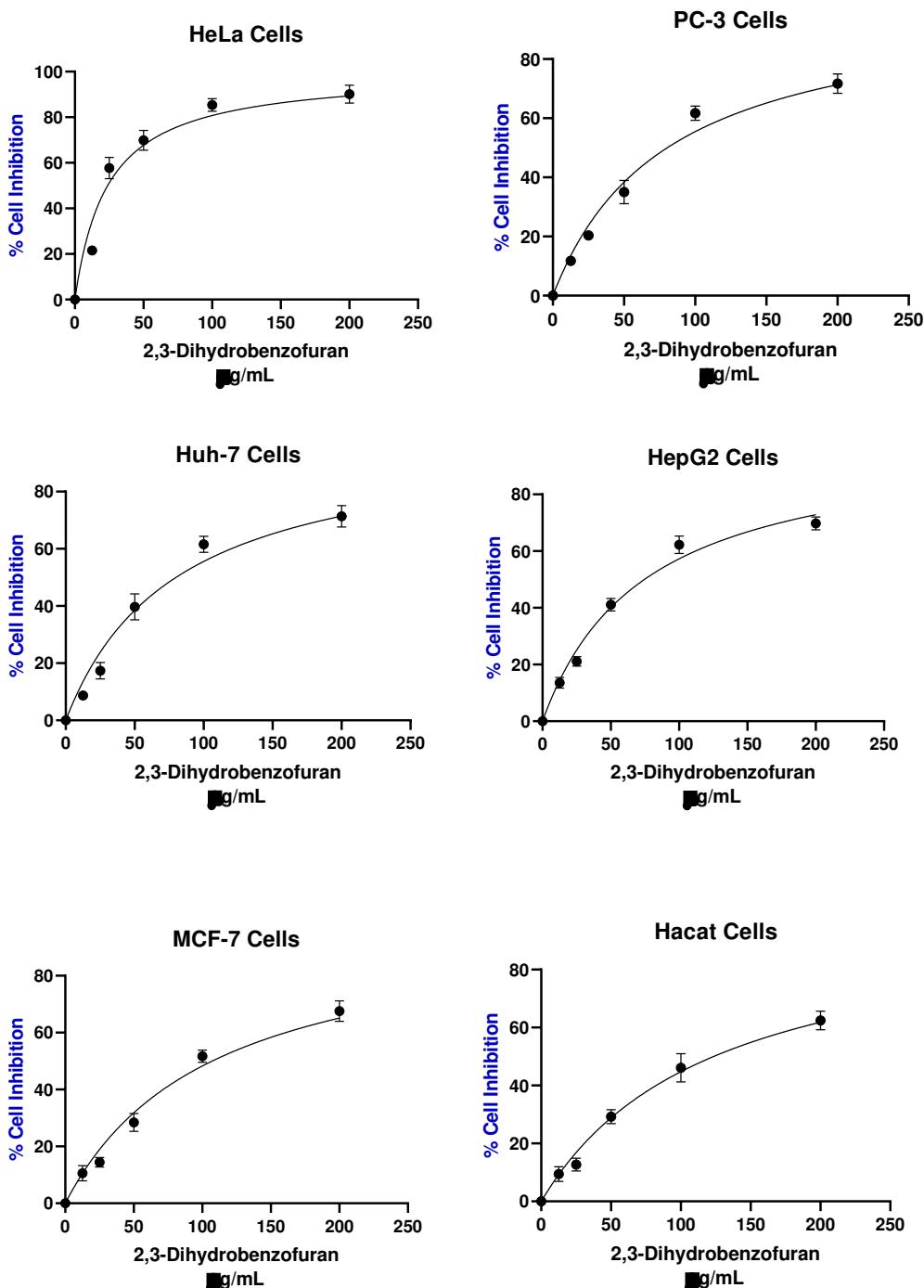


Figure 2. Results of the cytotoxic evaluation of 2,3-dihydrobenzofuran (7) in cancer cells.

In addition, is notable that HeLa cervical cancer cells were the most sensitive to the treatment of 2,3-Dihydrobenzofuran (7) (Table 2) with a IC_{50} of $23.86 \mu\text{g/mL}$ 3.97 times lower than IC_{50} of ethyl acetate extract of callus cultures of *A. pichinchensis*. This may suggest that the cytotoxic activity is associated with the effect of this compound, also a selective for cancer cells was also observed. The selectivity index is commonly reported in the literature as a ratio of IC_{50} values calculated for healthy and cancer cells [67,68] with values greater than 1 indicating desirable selectivity against cancer cells. The selectivity index of 2,3-dihydrobenzofuran (7) was 5.17, 1.53, 1.54, 1.65, 1.15 for HeLa, PC-3, Huh-7, HepG2, MCF-7 cells respectively.

Table 2. Cytotoxic activity of 2,3-dihydrobenzofuran (7) compound of *A. pichinchensis*.

Cellular line	IC ₅₀ µg/mL
HeLa	23.86 ± 2.5
PC-3	80.3 ± 4.6
Huh-7	79.9 ± 3.78
HepG2	74.62 ± 2.02
MCF7	107.2 ± 1.52
Hacat	123.5 ± 15.17

Previously, reported an important anti-inflammatory activity of 2,3-dihydrobenzofuran (7) [10], therefore, the results of our study reveal a relevant biological activity of the compound, with the advantage that its production is constant and controlled by the callus cultures of *A. pichinchensis*. This novel contribution shows that callus cultures represent a viable alternative for the production of bioactive compounds. It should be noted that the production of the compound has been improved in suspension cell cultures grown in flasks and airlift flasks [8,9].

3. Materials and Methods

3.1. Plant material from the wild plant

The plant species was collected in its natural habitat in the San Juan Tlacotenco neighborhood of the municipality of Tepoztlán in the state of Morelos, Mexico, and was identified by our research group in previously reported works and assigned the voucher number 39913. The specimen is under protection in the HUMO herbarium of the Autonomous University of the State of Morelos (UAEM), whose taxonomic identification was carried out by Biol. Gabriel Flores Franco [10].

3.2. Plant material from callus cultures

Calluses were previously established by our research group using leaf explants in Murashige and Skoog culture medium sterile (121 °C, 15 psi, for 15 min using an autoclave) supplemented with 30 g/L sucrose, 1 mg/L naphthaleneacetic acid, 0.1 mg/L kinetin, and 3 g/L phytogel [10]. Callus subcultures were carried out every 20 days and incubated at 25 ± 2 °C under photoperiod of 16 h with white fluorescent light (50 µmol / m² s).

3.3. Obtaining organic extracts

In Tepoztlán Morelos, 1465 g of fresh *A. pichinchensis* plant were collected, the plant was separated into leaves (910.73 g) and stems (415.76 g), these were dried under the shade at room temperature, finally obtaining leaves (145.18 g) and stems (78.13 g) dried. On another hand, 570.42 g of fresh biomass obtained from a callus culture of *A. pichinchensis* previously established were harvest, 13.20 g of dry biomass were obtained. The dry plant material (leaves and stem) and dry biomass (callus culture) were ground and extracted by maceration (72 hours, at room temperature) first with ethyl acetate and later with methanol (in triplicate). The solvent was removed by distillation under reduced pressure, using a rotary evaporator, finally obtaining 3 ethyl acetate extracts; leaves (17.20 g), stems (7.31 g) and callus culture (0.924 g) and 3 with methanol; leaves (59.09 g), stems (28.47 g) and callus culture (2.346 g)

3.4. Antiproliferative assay

PC-3 (prostate), HeLa (cervical), MCF7 (breast) and Huh-7 and HepG2 (hepatocellular) human cancer cell lines were purchased from ATCC (Manassas, VA, USA). We also included immortalized human epidermal keratinocyte line (Hacat) as a control of non-cancerous cells [69]. PC-3 was grown in RPMI-1640 medium (Sigma Aldrich, St. Louis, MO, USA), while Huh-7, HepG2, HeLa and Hacat in DMEM medium (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% SFB and 2 mM glutamine, all cultures were incubated at 37 °C in 5% CO₂.

Cells (4 × 10³ cells/well) were seeding in 96-well plates. The cells were treated with the investigated samples (at 200, 100, 50, 25 and 12.5 µg/mL) and incubated at 37 °C in 5% CO₂ for 48 h. Paclitaxel was used as positive control. For determining the number of viable cells in proliferation we

used CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), following the manufacturer's instructions. Cell viability was determined by absorbance at 450 nm using an automated ELISA reader (Promega, Madison, WI, USA). Stock solutions of all compounds were prepared in DMSO at a maximum concentration of 0.5%. The experiments were conducted by triplicate in three independent experiments. Data were analyzed in Prism 8.0 statistical program (Graphpad Software Inc., La Jolla, CA, USA) and the Half maximal inhibitory concentration (IC_{50}) were determined by regression analysis.

3.5. Purification of active compounds active ethyl acetate extract (Callus culture)

The chemical composition was determined by our research group in a previous study, which is why in this work we replicated the methodology and found reproducibility in the reported compounds [10], for which the technique of gas chromatography coupled with mass spectrometry was used that allowed the identification of the compounds in the present study, 2 mg of the extract were weighed and dissolved in 1 mL of chloroform to be analyzed by GC-MS (NIST 1.7a). The harvested calluses were dried in an oven at 40 °C, the dry biomass (14.76 g) was extracted three times with 100 ml of ethyl acetate by maceration; each extraction was every 72 hours at room temperature. The solvent was evaporated under reduced pressure and a yellow viscous extract (0.6859 g) was obtained. The ethyl acetate extract was fractionated on an open chromatographic column pre-packed with silica gel 18.76 g, (70-230 mesh; Merck) and eluted with a gradient system of n-hexane/ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70 and 00:100, v/v). 10 ml fractions were collected to obtain 45 fractions and monitored by TLC (ALUGRAM® SIL G/UV254 silica gel plates). The fractions that showed similarity in TLC were grouped obtaining 7 groups: GE-1 (1–16; 0.1802 mg), GE-2 (17–18; 15.6 mg), GE-3 (19–20; 78.3 mg), GE-4 (21–25; 40.8 mg), GE-5 (26–36; 81.6 mg), GE-6 (37–40; 70.6 mg), and GE-7 (41–45; 92.6 mg). The GE-1, GE-2 and GE-4 fractions were analyzed by GC-MS, this analysis indicated that the GE-1 fraction is made up of the mixture of n-hexadecanoic acid (1) and the ester of hexadecanoic acid (2), Fraction GE-2, by β -eudesmol acetate (3) and desmethoxyencecalin (4), purification of fraction GE-3 by column chromatography, using a gradient of n-hexane-ethyl acetate (100:00→70:30) provided 30 fractions. fractions 15–22 eluted with n-hexane-ethyl acetate (90:10) contained stigmasterol (5) and β -sitosterol (6), fractions 23–28 showed a single compound, identified by 1H and ^{13}C NMR and by comparison with their values reported in the literature [10] as (2S,3R)-5-acetyl-7,3-dihydroxy-2-(1-isopropenyl)-2,3-dihydrobenzofuran (15.3 mg, 7). The GE-4 fraction is made up of α -amyrin (8), epiluepol (9) and campesterol (10). Compound (11) was identified in the GE-5 fraction by direct comparison with an authentic sample available in the laboratory.

4. Conclusions

For the first time, it is shown that the chemical content of the ethyl acetate extract from *A. pichinchensis* callus cultures exhibits an important cytotoxic effect on the HeLa cell line, whose activity is suggested to be attributed to 2,3-dihydrobenzofuran, which has also shown anti-inflammatory effects. These results contribute to the development of alternatives for the treatment of cervical cancer, which has become a health problem that causes many deaths worldwide, particularly, this finding opens the possibility of new applications of callus culture extracts. of *A. pichinchensis* since the extracts of the wild plant did not show the cytotoxic effect. This is because in vitro cultures produce the compounds of interest in a constant and controlled manner, while wild plants depend on environmental, geographical, seasonal, among others.

Author Contributions: Conceptualization, M.S.-R., F.C.-S. and J.N.S.-C.; methodology, S.M.-B., J.N.S.-C. and J.G.E.-G.; software, E.C.-G. and F.C.-S.; validation, E.C.-G., J.G.E.-G. and F.C.-S.; formal analysis, V.D.-V and A.B.-A.; investigation, M.S.-R., J.G.E.-G. and F.C.-S.; resources, S.M.-B. and E.C.-G.; writing—original draft preparation, M.S.-R., F.C.-S. and J.N.S.-C.; writing—review and editing, S.M.-B., J.N.S.-C., J.G.E.-G., V.D.-V., A.B.-A., E.C.-G. and S.M.-B.; supervision, V.D.-V., F.C.-S. and A.B.-A.; funding acquisition, F.C.-S. and S.M.-B.

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