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In vitro anticancer screening of Colombian plants from *Piper* genus (Piperaceae)

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Historically, knowledge of ethnobotany, which has revealed different phytochemical and pharmacological compounds from traditional plants, has formed the basis for new anticancer drug discovery. The use of some *Piper* species in traditional medicine against cancer, suggests that genus *Piper* is a promising source of new compounds with anticancer activity. A total of 28 ethanolic extracts were obtained from 16 different *Piper* spp., then *in vitro* cytotoxicity activity was performed with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay in three certified human cancer cell lines (A549 lung, PC-3 prostate and MDAMB-231 breast). Seven ethanolic extracts obtained from different parts of *Piper eriopodon*, *Piper cumanense* and *Piper bogotense* showed promising anticancer effect with IC_{50} values below to 30 µg/mL. The most potent cytotoxic effect was found in the leaves ethanolic extract of *P. eriopodon* with an IC_{50} of 17.7 µg/mL for A549, 11.8 µg/mL for PC-3 and 20.7 µg/mL for MDAMB-231. Bioassay guided fractionation was performed for the most active extract and a highly cytotoxic compound was isolated and identified by spectroscopic means, mainly 1D and 2D RMN spectroscopy. The isolated compound identified as gibbilimbol B was shown to be a strong cytotoxic effect against cancer cell lines with IC_{50} values in the range of 11.4 and 41.9 µg/mL.

Key words: *Piper*, Piperaceae, alkenylphenols, cytotoxicity, anticancer, gibbilimbol B.

INTRODUCTION

According to the World Health Organization, cancer disease is a public health problem. By 2030, the number of people with cancer will reach 26.4 million in the whole world and now is considered one of the leading causes of death worldwide (WHO, 2014). Most of the drugs used in the pharmacological treatment of cancer disease are highly toxic and show low specificity to tumor cells, considerably affecting the survival prognosis of patients. Historically, natural product and ethnobotanical knowledge have been traditionally the main source of the discovery of new active principles that provide new horizons for cancer treatment (Cragg et al., 2014).

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However, many developing countries are still using traditional medicine due to their low cost and limited access to pharmacological treatments. The *Piper* genus is the most important genus of the Piperaceae family and recently was considered as potential source of new compounds with anticancer activity based on the use of some *Piper* species in traditional medicine (Sanubol et al., 2017; Calderón et al., 2006; Durant-Archibold et al., 2018; Mgbeahuruike et al., 2017). The vast majority of the global distribution for *Piper* spp. reside in the tropical zone of America (700 species) and Asia (300 species) (Jaramillo and Manos, 2001). In Colombia, the *Piper* genus is known as "cordoncillo" and is widely distributed in the tropical and humid forest, especially in the Chocó, Antioquia, Valle del Cauca, Santander and Cundinamarca regions.

Some of the *Piper* spp. reported in traditional medicine to treat cancer are *Piper aduncum, Piper boehmeriifolium* Wall*, Piper capense L.f., Piper* cubeba L., *Piper gibbilimbum* C.D.C., *Piper guineense* Schum and Thonn*, Piper longum* L., *Piper nigrum* L., *Piper sylvaticum* Rox*b* and *Piper barbatum* (Wang et al., 2014). Phytochemical reports on the genus *Piper* have shown that it contains a high amount of cytotoxic compounds, especially amide alkaloids (Bezerra et al., 2013; Meegan et al., 2017; Greenshields et al., 2015). Piperlongumine is a promising anticancer alkamide present in different plants of the *Piper* genus and it was shown to have selectively cytotoxic effect against cancer cells. The cytotoxic effect of piperlongumine in cancer cells, include induction of apoptotic cell death by cell cycle arrest in G1 or G2/M phase, increase of oxidative stress, inhibition of angiogenesis in xenograft-tumour mice model and destabilizing microtubules (Raj et al., 2011; Meegan et al., 2017). Other cytotoxic chemical constituents isolated from *Piper* plants are phenolic compounds such as phenylpropanoids (Ferreira et al., 2014; Hematpoor et al., 2018), flavonoids (Rossette et al., 2017; Niu et al., 2016; Freitas et al., 2014), lignans (Rajalekshmi et al., 2016; Sriwiriyajan et al., 2017) and alkenylphenols (Orjala et al., 1998; Lopes et al., 2013).

The present study investigated the *in vitro* cytotoxic effect of 28 ethanolic extracts, from 16 different Colombian *Piper* spp. against a panel of three human cancer cell lines. In order to explore the cytotoxic compounds, the chromatographic ultra-high performance liquid chromatography (UHPLC) profile of leaves, flowers and wood for the most active specie are presented. The majority component of the extracts was isolated, identified as an alkenylphenol and showed highly cytotoxicity activity.

MATERIALS AND METHODS

General experimental procedures

Flash chromatography was carried out with silica gel (230-400 mesh, Merck), analytical chromatography was performed using silica gel 60 PF254 (0.25 mm, Merck) and Shepadex® LH20 (Sigma). ^{1}H and ^{13}C NMR 1D and 2D were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C using the solvent peaks as internal references, the spectra were in $CDCl_3$ (δ_H 7.26 in ^{1}H and δ_C 77.0 in ^{13}C). High-resolution mass data were collected on an Accurate-Mass quadrupole Time-of-Flight (q-TOF) (Agilent Technologies) mass spectrometer, ESI positive mode, Nebulizer 50 (psi), Gas Flow 10 L/min, Gas Temp 350°C, Fragmentor 175 V, Skimmer 75 V, Vpp 750 V. UHPLC was performed on a Thermo Dionex UltiMate 3000 equipment, coupled with photodiode array (PDA) and evaporative light scattering detector (ELSD), using a Phenomenex Sinergy RP-C8 column (5 µm, 4.5 × 250 mm).

Plant

A total of 16 plants (Table 1) were collected mainly from the humid forest in Cundinamarca, Boyaca and Santander departments. The voucher specimens were deposited and identified at the Herbario Nacional Colombiano, Instituto de Ciencias Naturales, Universidad Nacional de Colombia by biologist Adolfo Jara Muñoz.

Preparation of the extracts

The plants were dried at room temperature protected from light. Then, the dried parts (e.g. leaves, wood or inflorescences) of the different plants were powdered and submitted to exhaustive extraction with ethanol 96% three times for 72 h at room temperature through a maceration process (Table 1). The ethanolic extracts were obtained after filtration through Wathman No. 1 filter and the evaporation of solvent with a rotatory vacuum evaporator at 40°C (Mesquita et al., 2009).

Cell culture

The human ATCC cell lines were cultured according to the ATCC protocols. Briefly, human cancer cells lines A549 lung cancer, PC-3 prostate cancer, MDAMB 231 and MCF7 breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin (Lonza) in a humidified atmosphere at 37°C in 5% CO_2.

In vitro cytotoxicity test

The cytotoxic effect of ethanolic extracts and isolated compound was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Riss et al., 2004). Briefly, cells were seeded in 96-well plates (10^4 cell/well) in 100 µL of medium supplemented with 10% FBS and 1% penicillin-streptomycin and allowed to settle for 24 h in a humidified atmosphere at 37°C in 5% CO_2. Then, the medium was removed and cells were treated with 100 µL of different concentrations (100, 30, 10, 1 and 0.1 µg/mL) of extracts or isolated compound prepared in cell culture medium. After 24 h, the treated medium of each well was removed and replaced by 100 µL of fresh medium containing 0.5 mg/mL of MTT and the plates were incubated for 4 h. Finally, the supernatant was removed and 100 µL of saline lysis buffer was added and measured in a Tecan Sunrise Eliza-Reader (Hombrechtikon, Switzerland) at λ = 595 nm. Untreated cells were used as negative control and the IC_{50} values (concentration that inhibits 50% of cell growth) of tested extracts and gibbilimbol B were calculated using GraphPad Prism software. The data is expressed as mean ± standard error of mean (SEM; n = 4).

Table 1. Plants and parts of plants used for the *in vitro* cytotoxic assay against the cancer cell lines: A549, PC-3 and MDA-MB-231.

Species	Voucher number	Plant part	IC_{50} (µg/mL)*		
			Lung A549	Prostate PC-3	Breast MDA-MB-231
Piper aduncum	COL595171	Leaves	>100	>100	>100
		Inflorescences	78.16 ± 10.7[a]	>100	>100
Piper amalago	COL 510519	Leaves	>100	>100	>100
Piper arboreum	COL 519815	Leaves	>100	>100	>100
		Wood	60.29 ± 7.22[b]	27.25 ± 2.08[a]	61.29 ± 3.65[a]
Piper arthante	COL 515965	Wood	>100	>100	>100
Piper cf. asperiusculum	COL 579924	Leaves	89.45 ± 10.3[c]	>100	>100
		Inflorescences	>100	>100	>100
Piper bogotense	COL 517696	Leaves	38.83 ± 3.89[de]	27.89 ± 2.44[b]	49.21 ± 5.22[b]
		Inflorescences	>100	>100	>100
Piper cumanense	COL 518183	Leaves	43.78 ± 3.23[e]	30.08 ± 1.59[b]	44.75 ± 1.85[c]
		Inflorescences	31.10 ± 2.56[f]	18.03 ± 1.35[c]	55.78 ± 4.07[d]
Piper el bancoanum	COL 518182	Leaves	>100	>100	>100
Piper eriocladum	COL 517694	Wood	>100	>100	>100
		Wood	>100	>100	>100
Piper cf. eriopodon	COL 516757	Leaves	17.84 ± 2.24[g]	11.88 ± 0.69[d]	20.75 ± 1.12[e]
		Inflorescences	33.74 ± 3.83[df]	16.90 ± 1.85[c]	53.22 ± 4.22[d]
		Wood	26.60 ± 1.85[f]	44.70 ± 3.70[e]	39.56 ± 2.51[f]
Piper hispidum	COL 510518	Leaves	>100	>100	>100
		Roots	>100	>100	>100
Piper holtonii	COL 517184	Wood	>100	>100	>100
		Leaves	>100	>100	>100
Piper marginatum	COL 591820	Wood	>100	>100	>100
		Leaves	>100	>100	>100
Piper peltatum	COL 512098	Leaves	>100	>100	>100
Piper pertomentellum	COL 579920	Leaves	>100	>100	>100
		Inflorescences	>100	>100	>100
Piper pesaresanum	COL 553307	Leaves	77.41 ± 4.92[g]	82.51 ± 4.75[f]	83.63 ± 7.23[g]

*****Values labeled with different letters are significant (Tuckey's HSD, $p < 0.05$).

UHPLC-DAD-ELSD analysis

The three most active extracts (leaves, inflorescences and wood of *Piper eriopodon*) were analyzed by liquid chromatography in order to determinate their complexity and similarities. 10 mg of each ethanolic extract were solubilized in 1 mL MeOH HPLC-grade. These solutions were filtered through 0.22 µm polytetrafluoroethylene (PTFE) membrane and placed in chromatography vials. The samples were analyzed by liquid chromatography (UHPLC) coupled with photodiode array (PDA) and evaporative light scattering detector (ELSD), in a Thermo Dionex UltiMate 3000 equipment. The separations were performed on a Phenomenex Sinergy RP-C8 column (5 µm, 4.5 × 250 mm). The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in gradient mode. The gradient started at 5% B for the first 5 min and increased linearly to 100% B over 25 min. It was kept constant

for 3 min and then returned to 5% B (30 min) and remained constant for 5 min. The flow rate was kept at 0.3 mL/min. The UV spectral was acquired between 200 and 400 nm. The retention times, peak areas and UV spectra of the major peaks were analyzed. Finally, the compound isolated from the leaves of *P. eriopodon* was examined under the same chromatographic conditions to dereplicate its presence in the other organs.

Isolation of cytotoxic compound

The leaves (1145 g) of *P. eriopodon* were extracted according to the procedure described earlier and solvent was evaporated to dryness resulting in 103.6 g of ethanolic extract. 100 g of extract were fractionated by flash chromatography on silica gel, eluted with a toluene/EtOAc in gradient mode (0 to 100% EtOAc) to obtain 8 fractions. The activity was retained in fractions 1 (34.2 g) and 2 (unpublished data). Fraction 1 (34.2 g) was submitted to silica gel chromatography eluted with a mixture of dichlorometane/ hexane/EtOAc (70:25:30) to obtain 10 fractions (P1-P10). The major component of fraction P5 (10 g) was submitted to a further purification by Sephadex LH-20 with a mixture (2:2:1) of hexane-chloroform-methanol to obtain the phenolic compound gibbilimbol B (7.93 g). The same process was used in air-dried wood (1490 g), which was obtained at 50.3 g of ethanolic extract and 768 mg of the same compound. The structure of isolated compound was identified by interpretation of the spectral data IR, HREIMS, ^{1}H and ^{13}C NMR (including DEPT, COSY, HMQC and HMBC experiments), as well as by comparison of the spectral data with those reported in the literature.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). The IC_{50} values were obtained by non-linear regression curve analysis of the concentration effect responses, using the GraphPad Prism program (Graph Software, San Diego, CA). Data for each cancer cell line were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD test. All differences with $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

A total of 28 ethanolic extracts were tested in the cytotoxicity assay. The species and the parts of plants used in the experiments, as well as the voucher number are shown in Table 1. Of the 28 extracts tested, it was found that 7 extracts showed strong cytotoxic activity against all tested cancer cell lines. These extracts were able to inhibit the growth of cancer cells in a dose-dependent relation. The IC_{50} values were calculated in a complete dose-response curve against three cancer cell lines A549, PC-3 and MDA-MB-231, the values are shown in Table 1. Among the 28 ethanolic extracts from Colombian *Piper* spp., 10 showed IC_{50} values lower than 100 µg/mL, 7 showed IC_{50} values lower than 50 µg/mL, and 3 values were lower than 20 µg/mL against the cancer cell lines tested.

The results showed that there are significant differences in the IC_{50} values for the cytotoxic extracts. Analysis of the data showed that the ethanolic extract from leaves of *P. eriopodon* has the most potent cytotoxic effect against the three human cancer cell lines tested, whereas the lowest cytotoxic effect was found in the ethanolic extract from wood of *Piper asperiusculum* (Table 1). The ethanolic extracts from leaves of *P. aduncum* and *P. asperiusculum* showed moderate anticancer activity only against lung cancer cells (A549). This cell line showed the most sensitive response, 10 ethanolic extracts inhibited the cell growth of lung cancer cells (A-549) with IC_{50} values lower than 100 µg/mL, in contrast to prostate (PC-3) and breast (MDA-MB-231) cancer cells, which eight of them showed cytotoxic activity lower than 100 µg/mL. The ethanolic extract of *P. aduncum* previously reported the presence of dillapiole, a phenolic compound isolated from many species of the *Piper* genus that induce apoptosis in cancer cells through the activation of caspases by the intrinsic pathway through the mitochondria (Ferreira et al., 2014). Other studies report that the ethanolic extract of leaves from *P. aduncum* has cytotoxic activities against HeLa cells (IC_{50}=3.91 µg/mL) and the dichloromethane extract against KB (IC_{50}=12 µg/mL) (Orjala et al., 1994), MCF7 (IC_{50}=27 µg/mL), H-460 (IC_{50}=25 µg/mL) and SF-268 (IC_{50}=23 µg/mL) cancer cells (Calderón et al., 2006), whereas *P. asperiusculum* have no previous reports.

According to the U.S. National Cancer Institute, an active extract is one with an IC_{50}<30 µg/mL (Suffness and Pezzuto, 1990). The present results showed that the ethanolic extracts from *P. eriopodon, P.cumanense and p.bogotense* have values of IC_{50} around or below to 30 µg/mL against human cancer cells, suggesting the presence of promising cytotoxic compounds in these *Piper* spp.

The cytotoxic activities of *P. eriopodon, P. cumanense, pesaresanum, P. bogotense* and *P. asperiusculum* against human cancer cell lines are reported for the first time in this study. The present data indicate that this ethanolic extracts are cytotoxic against human cancer cells and deserves further investigation in other cancer cell lines, as well as might be a potential source for the isolation of anticancer and chemotherapeutic agents.

The results showed that *P. eriopodon* has the most potent cytotoxic effect, specially the ethanolic extract from leaves with IC_{50} values of 11.88 µg/mL against prostate (PC-3), 17.84 µg/mL for breast (MDA-MB-231) and 20.75 µg/mL for lung (A549) cancer cells. In order to establish the presence of cytotoxic compounds, decision was made to study the three ethanolic extracts of *P. eriopodon* (leaves, wood and inflorescences) by liquid chromatography UHPLC to understand the complexity of the different extracts. The chromatographic profiles for the three ethanolic extracts were obtained in two detection systems, a first PDA profile measure at 254 nm and a second profile measure with an Evaporative Light Scattering Detector (ELSD) (Figure 1).

The UV chromatographic profiles showed higher complexity with many minority peaks at all retention times, mainly between 18 and 30 min which indicates a

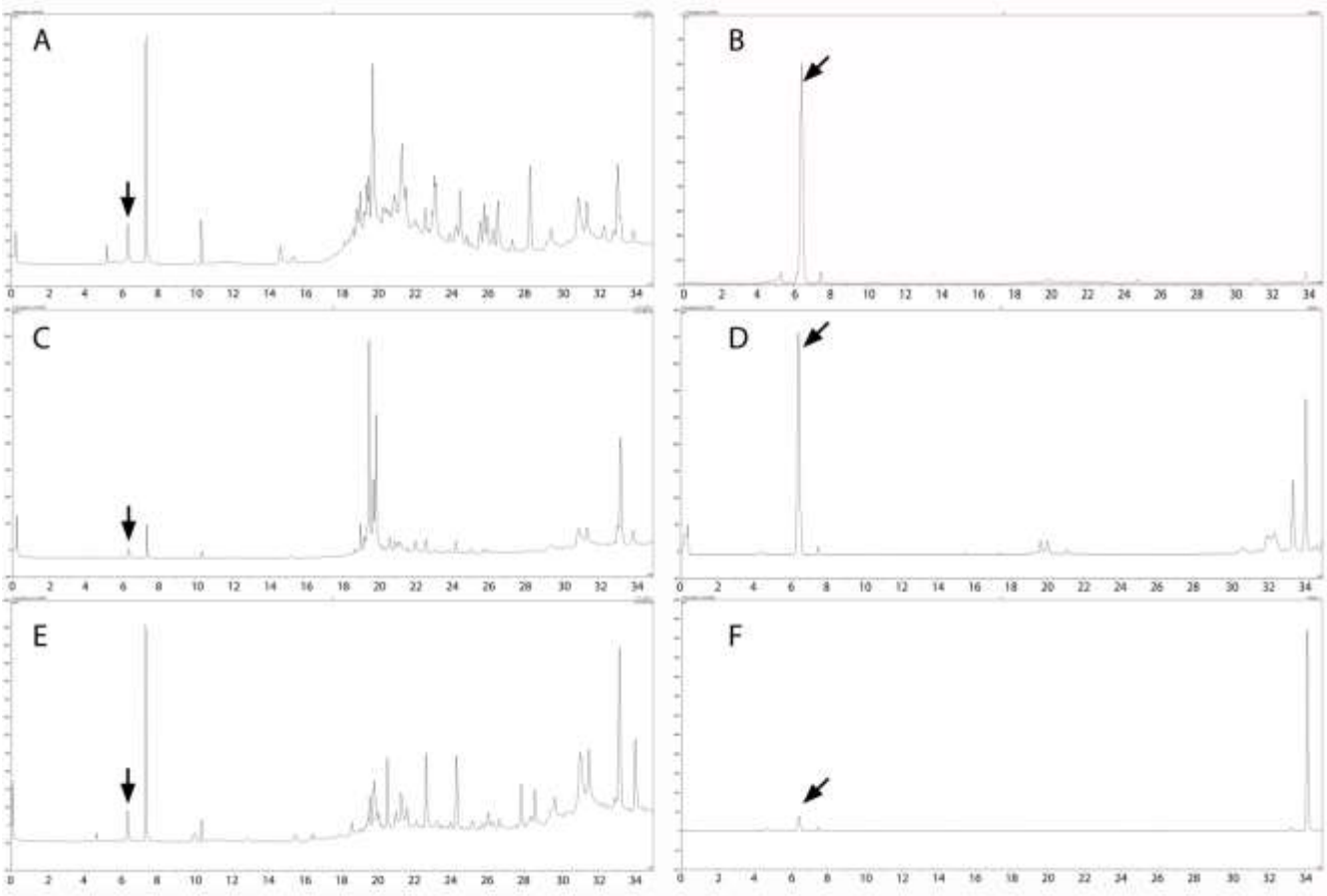

Figure 1. Chromatographic profiles at 254 nm for leaves (A), wood (C), inflorescences (E) and profiles with ELSD detector system for leaves (B), wood (D) and inflorescences (F) of the ethanolic extracts from *P. eriopodon*. The *X* axis corresponds to retention time in minutes and black arrow in all chromatograms corresponds to isolated compound gibbilimbol B.

massive presence of nonpolar metabolites. In the wood extract, the presence of two high peaks close to 20 min was observed, which was not current in the other extracts. In contrast, the chromatographic ELSD profiles for all extracts (Figure 1) showed low complexity, especially in leaves and wood, where almost a single peak is noted at 6.50 min. This observation can be considered indicative of large amounts of one compound in the ethanolic extracts. The ELSD detector response is related to the absolute quantity of analyte present, significantly different to UV detector, in which the analyte must have a chromophore, resulting in a signal completely dependent of the compound spectral properties (Swartz, 2010). The results, clearly showed the large presence of one compound in the ethanolic extracts of leaves and wood, followed by a smaller amount in inflorescences from *P. eriopodon*.

To establish the relation between this compound and the cytotoxic effect of *P. eriopodon*, the ethanolic extracts were subjected to a further purification, as described in the experimental section. The isolated compound was obtained from leaves in large amounts (7.93 g) and identified as an alkenylphenol named gibbilimbol B. The chemical structure of gibbilimbol B was elucidated completely by the interpretation of the spectral data and the comparison with those reported in the literature (Figure 2) (Orjala et al., 1998). The presence of gibbilimbol B was verified through comparison of chromatograms in all extracts, mainly in leaves, wood and in a smaller proportion in the inflorescences; which might be related to the greater cytotoxic activity of the ethanolic extract of leaves from *P. eriopodon* against cancer cells. Previously, this alkenylphenol had shown cytotoxic activity in KB carcinoma cells (ED_{50} = 3.9 ug/mL) (Orjala et al., 1998). Thus, was decided to evaluate the cytotoxic effect of gibbilimbol B against A549, PC-3, MDAMB-231 and MCF7 cells. The present results confirm that gibbilimbol B is effective in suppressing cancer cells growth in a dose-dependent manner, with IC_{50} values of 39.7, 32.1, 11.1 and 11.9 µg/mL for A549, PC-3, MDAMB-231 and MCF7 cells, respectively (Figure 3).

Gibbilimbol B was isolated previously in small quantities from the medicinal plant *P. gibbilimbum*, a

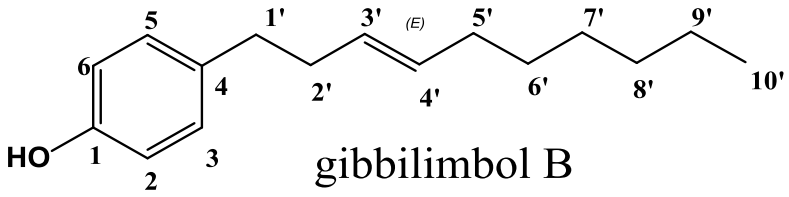

Figure 2. Chemical structure of isolated compound.

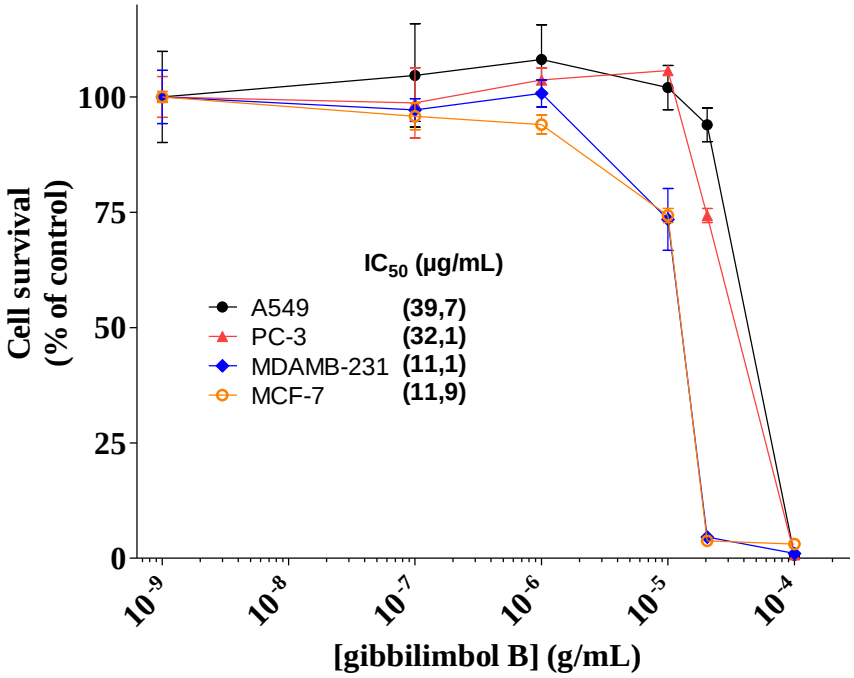

Figure 3. Inhibition of cell growth by gibbilimbol B and IC_{50} values for lung (A549), prostate (PC-3) and breast (MDAMB-231 and MCF7) cancer cells. The data is expressed as mean ± SEM (n = 4).

native plant from Papua New Guinea used in traditional medicine to treat fever, abscesses, ulceration of the skin and the juice from the bark is taken by patients with suspected cancer (Worth and Kerenga, 1987). Recently, this alkenylphenol was isolated too in small quantities from *Piper malacophyllum* and showed promising antitrypanosomal activity against *Trypanosoma cruzi*, with an EC_{50} of 17.49 µg/mL (de Oliveira et al., 2012; Varela et al., 2016). However, the limited amount of gibbilimbol B in nature restrict the possibility to perform new biological studies, prompted in scientists the interest to search a different synthetic strategies to obtain more quantities of this compound (Abe et al., 2001; Vyvyan et al., 2002; Zhou et al., 2004; Wang et al., 2009; Varela et al., 2016). The present results clearly show that gibbilimbol B is the majority component of the leaves from *P. eriopodon*, corresponding to 8% of the total ethanolic extract, indicating that *P. eriopodon* can be considered for further investigation as a natural source to obtain easily and in large amounts this bioactive alkenylphenol.

Some alkenylphenols reported as promising anticancer agents are 4-nerolidylcatechol and climacostol. 4-Nerolidylcatechol was isolated from *Piper umbellata* and induces apoptosis in SK-Mel-28 (melanoma) and in multidrug-resistant human chronic myeloid leukemia K562 cells with an IC_{50} of 24.5 µM (Cortez et al., 2015; Benfica et al., 2017). Climacostol is another potent cytotoxic alkenylphenol produced by the ciliated protozoan *Climacostomum virens* that inhibits selectively

the growth of tumor cells and induces apoptosis in cancer cells *in vitro* and *in vivo* assays (Buonanno et al., 2008; Perrotta et al., 2016).

Regarding to the IC_{50} values in cell lines tested, it was found that gibbilimbol B have partial selectivity for breast cancer cells MDAMB-231 and MCF7. The genetic differences between those cells lines include that MCF7 is estrogen and progesterone receptor positive (ER+ and PR+), HER2 negative (HER2-) and P53 wild-type, while MDAMB-231 cell line is triple-negative breast cancer (ER-/PR-/HER2-) and P53 mutant, an aggressive form of breast cancer with limited treatment options (Hahm and Singh, 2013; Neve et al., 2006). Because differences in IC_{50} values are not significant, the present study results suggest that the molecular basis implicated in the cell death are hormone independent and may be more associated with the inhibition of common pathways related to promote the oncogenic activities like cell cycle progression, senescence and metastasis in both cell lines as the PI3K/AKT pathway (Lin et al., 2009; Gao et al., 2009) or even the inhibition of the high expression levels of XIAP reported for both cell lines (Hahm and Singh, 2013; Nikolovska-Coleska et al., 2004; Obexer and Ausserlechner, 2014).

In conclusion, the findings of current study showed that *P. eriopodon* extracts present the highest cytotoxic activity among the others *Piper* selected Colombian plants. The alkenylphenol gibbilimbol B is the majority component in *P. eriopodon* and the results of the present study showed that gibbilimbol B present highly cytotoxic activity in human cancer cells, partially selective for breast cancer cells MCF7 and MDAMB-231. Though molecular basis involve in the triple-negative breast cancer is unclear, the findings of this study show that gibbilimbol B is a promising cytotoxic compound and is crucial in the future to perform molecular analysis to understand the mechanism involve in the induced cell death mediated by this alkenylphenol.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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