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*Full Length Research Paper*

# Cytotoxic effect of *Plectranthus neochilus* extracts in head and neck carcinoma cell lines

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**Following a tendency of studying the potential effects of plant extracts to cancer, this study aimed to evaluate *in vitro* the cytotoxic activity of *Plectranthus neochilus* (PN) extracts and its fractions in head and neck squamous cell carcinoma (HNSCC) cell lines and assess their tumor specificity. MTT assay was conducted with two HNSCC cell lines, FaDu (hypopharynx carcinoma) and SCC-25 (tongue carcinoma), one keratinocyte (HaCat) and one fibroblast (L929) cell line. Two PN leaf crude extracts, one ethanolic (E) and one hexanic (H), and their nine fractions were tested. A dose-response curve was performed with hexane PNH fraction and a tumor specificity index (TSI) was calculated. For all cell lines studied, almost all extracts and fractions resulted in cell viability lower than 50%. Hexane and methanol PNH fractions were exceptions, causing a significantly low viability in SCC-25 (17.16 and 34.53%, respectively), but higher than 50% in FaDu, HaCat and L929. The dose-response curve with hexane PNH fraction resulted in a $CC_{50}$ of 540.9 µg/mL for FaDu, 550 µg/mL for L929, 762.1 µg/mL for HaCat and 274.2 µg/mL for SCC-25. The TSI L929/FaDu was 1.01, HaCat/FaDu was 1.40, L929/SCC-25 was 2.00 and HaCat/SCC-25 was 2.77. TSIs indicate its specificity for tongue carcinoma cells, when compared to fibroblasts and keratinocytes.**

**Key words:** Head and neck, squamous cell carcinoma, extract, cytotoxicity, cell line.

## INTRODUCTION

Cancer is one of the most common causes of morbidity and mortality today, being the cause of 8.2 million deaths in 2012 (World Health Organization (WHO), 2014). Such number is predicted to increase in the next years throughout the world, with an incidence of 14 million, as calculated in 2012, and estimated to rise up to 22 million in the next two decades (International Agency for Research on Cancer (IARC), 2012). Moreover, two thirds of all cancer diagnostics occur in low- and middle-income countries (WHO, 2014).

Head and neck cancer figures as the sixth most common type of cancer worldwide, being specially incident in the south and southeast of Asia, parts of Europe, and parts of South America (Warnakulasuriya, 2009). It is traditionally associated with tobacco and alcohol consumption, and more recently, the importance

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of human papillomavirus (HPV) as an etiological factor has been evidenced, mainly for oropharyngeal cancer (Pytynia et al., 2014; Thavaraj et al., 2011). According to the National Cancer Institute in the United States, the occurrence of 42,440 new head and neck cancer cases was estimated for 2014, as well as 8,390 deaths due to the disease (National Cancer Institute (NCI), 2014).

The treatment procedures depend on the stage of the disease, and surgery associated to radiotherapy and/or chemotherapy happens to be the treatment of choice in most situations. Obviously, each case ought to be analyzed independently, given that the morbidity of the surgical resection in the oral cavity is considerable and surgery must be avoided whenever possible (Belcher et al., 2014; Omura, 2014). Such idea instigates the search for new therapeutic options that are less deleterious to the patient's general health status than the surgery, radio and chemotherapy regimens prescribed currently.

The genus *Plectranthus* (Lamiaceae) comprises about 300 species distributed through the tropical and warm regions of the Old World, Africa, India and Australia (Rice et al., 2011). In Brazil, plants of this genus are widely used in folk medicine, mainly for digestive disturbances and liver complains (Bandeira et al., 2010; Lukhoba et al., 2006). The main phytochemical constituents of the genus *Plectranthus* are diterpenoids, essential oil and phenolics (Abdel-Mogib et al., 2002). However, concerning chemical composition for several species, little is still well known.

One of those, *Plectranthus neochilus*, called "boldinho", "boldo-da-folha-miúda" or "boldo-gambá" (Duarte and Lopes, 2007), beside digestive disturbance, is also used for pain, edema, skin infections and respiratory ailments (Caixeta et al., 2011; Madaleno, 2011; Moreira et al., 2002). In South Africa, an ethnomedicine survey revealed this species is used for treating respiratory infections (York et al., 2011). Little information may be found on its chemical composition. The essential oil composition is well reported, but can vary depending on several factors, for example, geographic differences, if the extraction occurs using fresh or dried plant material, and others (Caixeta et al., 2011; Lawal et al., 2010; Rosal et al., 2011). Essential oil from fresh leaves of *P. neochilus* from the southeastern region of Brazil presented β-caryophyllene, α−thujene and α-pinene as main compounds (Caixeta et al., 2011; Rosal et al., 2011), while a sample from South Africa showed citronellol and citronellyl formate as major compounds (Lawal et al., 2010). Hexane extract from leaves and stems furnished friedelin, fatty acid ester of α-amyrin, sitosterol and stigmasterol, while flavone cirsimaritin could be isolated from ethanol extract (Viana, 2011). In the same way, there are few references concerning biological activity of this species. The essential oil of leaves (100 µg/mL) presented activity against *Schistosoma mansoni*, killing 100% of adult worms (Caixeta et al., 2011). The essential oil causes the separation of male from female, deflecting the establishment of the infection. Cellular viability test using V79 cells (lung tissue of young male hamster) resulted in no cytotoxicity at doses lower than 200 µg/mL (Caixeta et al., 2011).

Essential oil from *P. neochilus*, presenting α-terpenyl acetate, α-athujene, β- caryophyllene, β-pinene, and α-pinene as major volatile components, was assayed with the thiobarbituric acid reactive substances (TBARS) test to evaluate the capacity for preventing lipid oxidation. As a result, a fairly anti-oxidant activity was observed only at high concentration (1.0 g/L), in comparison to α−tocopherol (Mota et al., 2014).

Methanol extract from leaves was tested against *Leishmania amazonensis* and *L. (L.) chagasi* and did not show any activity (Tempone et al., 2008). However, when tested against fluconazole-resistant *Candida krusei* (ATCC 6528) and the azole-susceptible *Candida parapsilosis* (ATCC 22019), it showed activity against *C. krusei* ($EC_{50}$ = 20.51 µg/mL) (Tempone et al., 2008).

An ointment prepared with hydroethanol extract from leaves of *P. neochilus* as active component showed analgesic activity in female cats in post-surgery pain (Silva et al., 2013). Because *P. neochilus* is widely spread in Brazil, the aim of this study was to evaluate the potential cytotoxicity of crude extracts and fractions against head and neck squamous cell carcinoma cell lines and to assess the tumor specificity of these extracts by comparing their activity on cancer cells to their results on control cells.

## MATERIALS AND METHODS

### Plant

Aerial parts of *P. neochilus* were collected at Campus Universitario Darcy Ribeiro, Universidade de Brasília in January, 2011. A voucher species was deposited at Herbarium of Universidade de Brasilia (UB) (voucher number S. M. Gomes & P. Monteiro nº 913. After separation, leaves (979.0 g) were dried over temperature lower than 40°C and then submitted to extraction, by passive maceration technique, first using hexane, followed by ethanol. After solvent elimination under vacuum and temperature lower than 40°C, crude hexane extract (PNH, 5.1% yield) and crude ethanol extract (PNE, 7.5% yield) were obtained. Part of PNH (20.0 g) was submitted to silica gel 60 G Merck filtration (silica layer: high 5.1 cm, diameter 7.9 cm) furnishing 5 fractions: FHex (7.0 g); F (Hex:AcOEt) (12.0 g); FAcOEt (0.5 g); F (AcOEt:MeOH) (0.6 g); FMeOH (0.1 g). Part of PNE (6.0 g) was submitted to liquid-liquid partition and solvent extraction, resulting in 4 fractions: FrHex (1.0 g); $FrCH_2Cl_2$ (1.1 g); FrAcOEt (2.3 g); FrH2O (1.3 g). Crude extracts and fractions were submitted to cytotoxicity assay.

### Cell lines and culture conditions

Human head and neck cancer cell lines, one of tongue squamous cell carcinoma (SCC-25) and another of hypopharyngeal carcinoma (FaDu) were used. A keratinocyte cell line (HaCat) and a fibroblast cell line (L929) were used as cell control. For the culture of SCC-25, cells were grown as monolayers in a mixture of Dulbecco's modified eagle medium (DMEM) and Ham's F12 in a proportion of 1:1, and supplemented with 10% fetal bovine serum and 1% antibiotics

**Table 1.** Evaluation of cell viability after treatment with *Plectranthus neochilus* crude extracts and their fractions.

| Extracts and f ractions | Sample | No. | Viable cells (%) | | | |
|---|---|---|---|---|---|---|
| | | | FaDu | SCC-25 | L929 | HaCat |
| *Plectranthus neochilus* ethanolic crude extract | PNE | 1 | 15.54 | 26.29 | 14.64 | 21.47 |
| *Plectranthus neochilus* hexanic crude extract | PNH | 2 | 6.77 | 31.44 | 16.38 | 6.12 |
| Dichloromethane fraction from PNE | $FrCH_2Cl_2$ | 3 | 17.04 | 28.00 | 13.17 | 21.44 |
| Hexane fraction from PNE | FrHex | 4 | 4.59 | 39.41 | 11.28 | 5.02 |
| Aqueous fraction from PNE | FrH2O | 5 | 28.99 | 30.60 | 18.54 | 20.10 |
| Ethyl acetate fraction from PNE | FrAcOEt | 6 | 29.72 | 26.18 | 18.17 | 22.78 |
| Ethyl acetate fraction from PNH | FAcOEt | 7 | 9.66 | 10.13 | 7.23 | 6.98 |
| Hexane fraction from PNH | FHex | 8 | 84.72 | 17.16 | 69.95 | 82.29 |
| Ethyl acetate:methanol (1:1) fraction from PNH | F(AcOEt:MeOH) | 9 | 9.76 | 19.84 | 13.27 | 8.50 |
| Hexane:ethyl acetate (1:1) fraction from PNH | F(Hex:AcOEt) | 10 | 6.97 | 8.84 | 7.23 | 7.44 |
| Methanol f raction f romPNH | FMeOH | 11 | 63.45 | 34.53 | 52.36 | 62.77 |
| Positive control | Cisplatin | - | 50.38 | 36.55 | 31.24 | 36.51 |
| Negative control | DMSO/Ethanol 2:3 | - | 100 | 100 | 100 | 100 |

Tongue carcinoma (SCC-25), Hypopharyngeal carcinoma (FaDu), Keratinocyte (HaCat) and Fibroblast (L929) cell lines. 1. *Plectranthus neochilus* ethanolic crude extract (PNE); 2. *Plectranthus neochilus*hexanic crude extract (PNH); 3. Dichloromethane fraction from PNE (FrCH2Cl2); 4. Hexane fraction fromPNE (FrHex); 5. Aqueous fraction from PNE (FrH2O); 6. Ethyl acetate fraction from PNE (FrAcOEt); 7.Ethyl acetate fraction from PNH (FAcOEt); 8. Hexane fraction from PNH (FHex); 9. Ethylacetate:methanol (1:1) fraction from PNH [F(AcOEt:MeOH)]; 10. Hexane:ethyl acetate (1:1) fraction fromPNH [F(Hex:AcOEt)]; 11. Methanol fraction from PNH (FMeOH).

(penicillin-streptomycin). HaCat, FaDu and L929 were cultured in DMEM with the same supplements described before. Cells were maintained at 37°C and 5% of $CO_2$. For all experiments, cells were detached with trypsin (0.25%)/EDTA (1 mM) solution. All cell culture reagents were purchased from Sigma-Aldrich (ET. Louis, MO).

#### Cell viability assay (MTT)

Cells were seeded at the density of $5 \times 10^3$ cells/well in a 96-well plate and then treated with *P. neochilus* extracts and its fractions (Table 1), at 500.0 µg/mL. After 24 h of treatment, cell viability was assessed by MTT assay. This test assesses the ability of mitochondrial enzymes of treated cells to convert tetrazolium salts (MTT) (3-[4,5- dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) in formazan, so only viable cells, or cells that have not undergone sufficient damage to reduce their mitochondrial activity, have the ability to accomplish this reduction. Then, the absorbance was measured and the values obtained with treated cells were compared to the values of cells treated with control. For this assay, 10.0 µL MTT solution (5 mg/mL) (Sigma) were added to each well, followed by incubation for 4 h at 37°C. After incubation the medium was discarded and formazan crystals were dissolved in 100 µL acidified isopropanol solution (25.0 mL isopropanol added of 104.0 µL HCl) and vortexed in low velocity for 15 min. Absorbance was measured at 570 nm in a Beckman Counter reader. Treatment with Cisplatin (50.0 µg/mL) was used as a positive control, and as negative control the solvent used with the extracts and fractions, which was dimethyl sulphoxide (DMSO): Ethanol (2:3). Experiments were carried out at least three independent times and were performed in triplicates.

#### Dose-response curve

HaCat, L-929, FaDu, and SCC-25 cells were seeded as described earlier for the cytotoxicity assay and incubated overnight in ideal conditions. Cells were then treated with serial dilutions of hexane PNH fraction in decreasing concentrations (1000.0, 750.0, 500.0, 250.0, 125.0 µg/mL) or vehicle control. After 24 h of treatment, MTT assay was performed as described. Graphpad Prisma 5.0 was used to define the 50% cytotoxic concentration ($CC_{50}$) of tested extracts. The $IC_{50}$ values were used to calculate the tumor specificity index (TSI) for each treatment, calculated as the ratio of the $IC_{50}$ of control cell lines and cancer cell lines, as proposed by Horii et al. (2012). The TSI equal to 1 means no selectivity between cell lines, TSI less than 1 means the treatment is more selective for control cell lines than for cancer cell lines and TSI greater than 1, it means that there is selectivity for the cancer cell line studied. The TSI was calculated according to the equation: TSI = ($CC_{50}$ control cell) / ($CC_{50}$ cancer cell) (Horii et al., 2012). L929 and HaCat were considered control cell lines and FaDu and SCC-25 were the cancer cell lines.

#### Statistical analyses of data

Statistical analysis was performed on the means of triplicates that resulted from at least three independent replications of all experiments. All data were analyzed using GraphPad Prisma 5.0. For cytotoxicity assay, one way analysis of variance (ANOVA) was used, with Tukey's multiple comparison test as a post-test. In dose response curve was used nonlinear regression, variable slope with log inhibitor *versus* response.

## RESULTS AND DISCUSSION

The crude ethanol and hexane *P. neochilus* extracts (PNE and PNH, respectively) resulted in statistically significant viability reduction in all cell lines studied, which was always inferior to 50% of the cell viability compared with negative control (considered as 100% of viable cells), as seen in Figure 1 and Table 1. Such viability reduction was especially intense for the hypopharyngeal

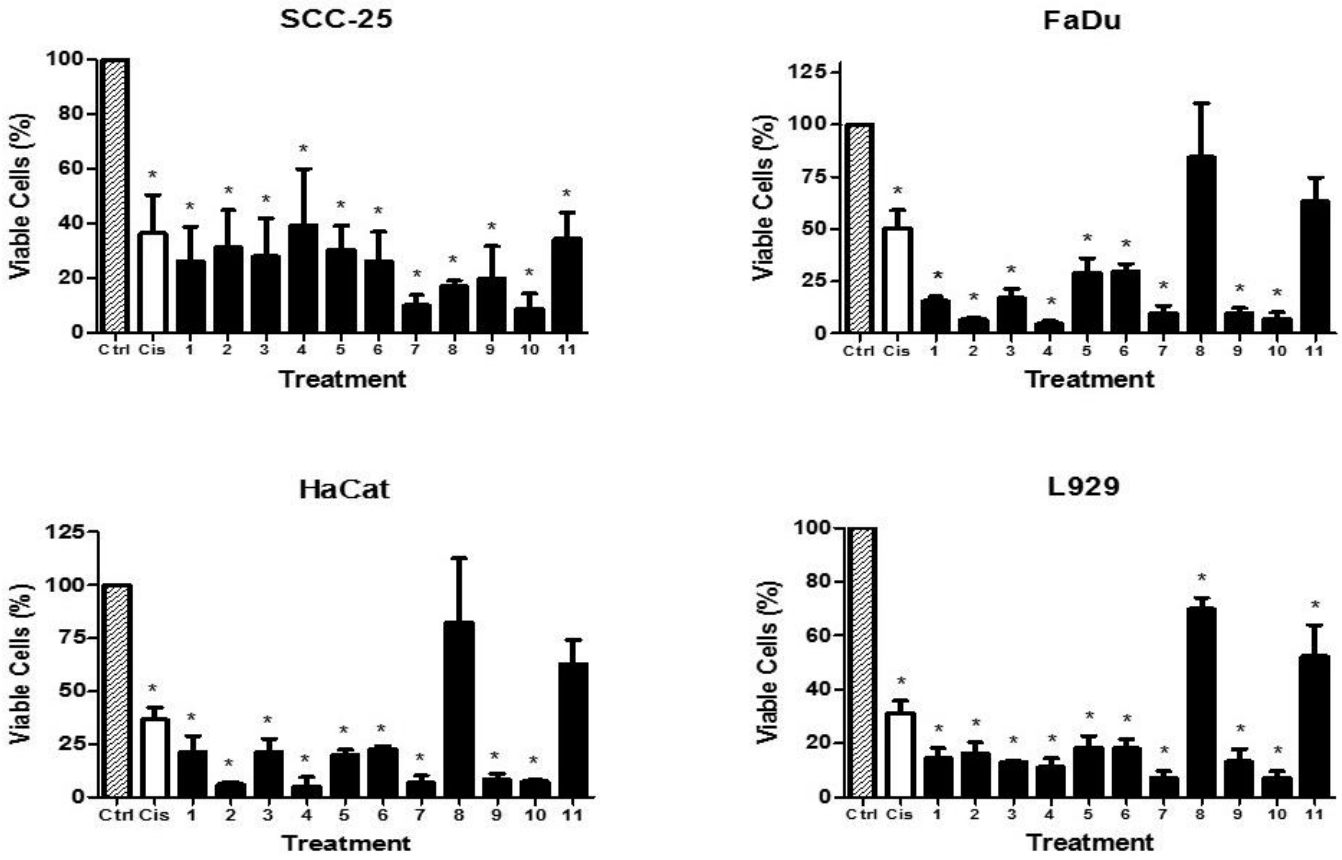


**Figure 1.** Cell viability after a 24 h-treatment with extracts and fractions. Tongue carcinoma (SCC-25), Hypopharyngeal carcinoma (FaDu), Keratinocyte (HaCat) and Fibroblast (L929) cell lines. Ctrl = Control (DMSO/Ethanol 2:3); Cis = Cisplatin (50 µg/mL); 1. *Plectranthus neochilus* ethanolic crude extract (PNE); 2. *Plectranthus neochilus* hexanic crude extract (PNH); 3. Dichloromethane fraction from PNE ($FrCH_2Cl_2$); 4. Hexane fraction from PNE (FrHex); 5. Aqueous fraction from PNE ($FrH_2O$); 6. Ethyl acetate fraction from PNE (FrAcOEt); 7. Ethyl acetate fraction from PNH (FAcOEt); 8. Hexane fraction from PNH (FHex); 9. Ethyl acetate:methanol (1:1) fraction from PNH [F(AcOEt:MeOH)]; 10. Hexane:ethyl acetate (1:1) fraction from PNH [F(Hex:AcOEt)]; 11. Methanol fraction from PNH (FMeOH). The results are representative of at least three independent experiments and show the mean±SEM.*$p$<0.05 *vs* control.

carcinoma cell line (FaDu), which resulted in a viability of 15.54% with PNE and 6.77% with PNH, for fibroblasts cells (L929), with a resultant viability of 14.64 and 16.38% for PNE and PNH, respectively, and for the keratinocytes cells (HaCat), with a viability of 21.47% for PNE and 6.12% for PNH. A viability of 26.29 and 31.44% could be observed in the tongue oral squamous cell carcinoma cell line (SCC-25) for PNE and PNH. Whenever compared to the positive treatment control, Cisplatin treatment with extracts was not considered statistically different, although the extracts seemed to be more cytotoxic than Cisplatin for all cell lines, given that they resulted in lower cell viability levels (Table 1).

The four PNE fractions (dichloromethane, hexane, aqueous and ethyl acetate fractions) and three of the PNH fractions (ethyl acetate, ethyl acetate:methanol and hexane:ethyl acetate fractions) were found to be highly cytotoxic to all tested cell lines, resulting in cell viability rates lower than 30% for FaDu (from 6 to 29%), HaCat (from 5 to 21%) and L-929 cells (from 7 to 18%), and lower than 40% for SCC-25 (from 8 to 39%) (Table 1). When compared to Cisplatin, these fractions were considered equally or more cytotoxic. Treatment with fractions resulted in a more intense cell cytotoxicity to HaCat and L929 than to tongue carcinoma cell line.

Different results might be observed for the hexane and methanol PNH fractions (FHex and FMeOH, respectively). FHex lead to a cell viability of 84.72% in FaDu, 69.95% in L929 and 82.29% in HaCat, while FMeOH fraction resulted in a viability of 63.45% with FaDu cells, 52.36% with L929 cells and 62.77% with HaCat cells. These cell viability rates were considerably higher than those observed after treatment with other fractions, the crude extracts and even the positive control. Interestingly, these fractions did not cause the tongue cancer (SCC-25) cell line such a small reduction in cell viability, when compared to Cisplatin or the other treatment regimens. In the tongue carcinoma cells, FHex

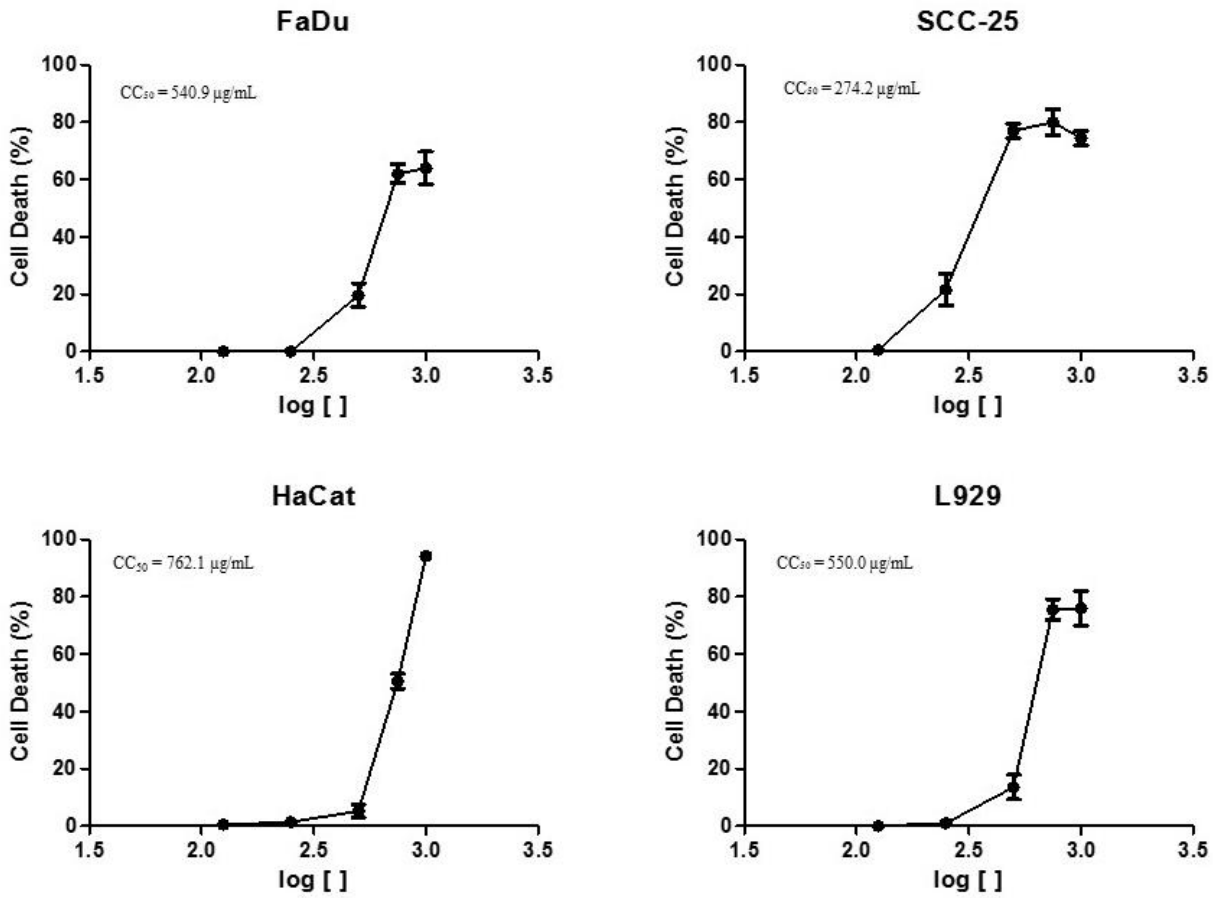


**Figure 2.** Hexane fraction from *Plectranthus neochilus* hexanic crude extracts (8. FHex) dose-response curves. CC50 = 50% Cytotoxic Concentration.Tongue carcinoma (SCC-25), Hypopharyngeal carcinoma (FaDu), Keratinocyte (HaCat) and Fibroblast (L929) cell lines.

caused a viability rate of 17.16% and a 34.53% rate was observed with FMeOH. Cisplatin resulted in a viability of 36.55% in this cell line.

Both FHex and FMeOH fractions were considered to be of special interest, given that they seemed to be highly cytotoxic to the tongue carcinoma cells and considerably less cytotoxic to the control fibroblast and keratinocyte cells. To further investigate and evaluate this potentially specific cytotoxic effect observed in this initial viability assay, dose-response curves were performed with the FHex fraction, for its cell viability result were lower with SCC-25 cells and higher with L929 and HaCat cells, when compared to the results observed with FMeOH fraction. The FHex fraction dose-response curves may be seen in Figure 2. A $CC_{50}$ was calculated for each cell line. The doses of 540.9 µg/mL for FaDu, 550.0 µg/mL for L929 cells and 762.1 µg/mL for HaCat cells were calculated as the FHex $CC_{50}$ in the respective cell lines. A smaller $CC_{50}$, 274.2 µg/mL, was found for SCC-25 cells, all in accordance with the results found in the initial cell viability assay.

The TSI was calculated on these values of $CC_{50}$. The TSI FaDu × L929 cells was 1.02 and the FaDu × HaCat was 1.41, which means that the fraction was not selective for the hypopharyngeal carcinoma cells, being equally cytotoxic to the cancer cells, fibroblasts and a little less toxic for keratinocytes. The TSI SCC-25 × L929 cells was 2.01 and the SCC-25 × HaCat cells was 2.78. These index values indicate a specificity of FHex for the tongue carcinoma cells (SCC-25), being the highest tumor specificity observed when SCC-25 and HaCat were analyzed. Similar results were already described in a study that found specific non-apoptotic cell death in oral squamous cancer cells induced by *Rhinacanthus nasutus* extracts (Horii et al., 2012). Interestingly, the specificity for cancerous cells was more evident when SCC-25 cells were taken into consideration, if compared to what could be observed with hypopharyngeal carcinoma cells (FaDu). Although both cell lines were derived from tumors that originate from a common cell type, the epithelial squamous cell, alterations and mutations in genes and molecular pathways vary in different tumors

(Chatelain et al., 2011). The genetic heterogeneity might justify the difference in results with SCC-25 and FaDu cell lines whenever they were submitted to the same treatment regime.

Conventional therapeutic approaches for HNSCC include surgery, radiotherapy and chemotherapy used alone or in combination. All therapies are associated with many adverse effects, such as post-surgical facial disfiguration or acute and chronic toxicities. Consequently, chemotherapy and radiotherapy reduce patient's quality of life. Many of these side effects are caused by the lack of selectivity of conventional therapies, which target both normal and cancerous cells. In this sense, the search for more new selective and effective therapies is highly required (Du et al., 2014).

Plants are major suppliers of substances active against cancer. For the first time, the cytotoxic activity of *P. neochilus* leaves extracts on HNSCC cell lines was reported. It is interesting to observe that, in general, the less polar fractions showed the most significant activity. A phytochemical analysis of PNH (unpublished results) characterized the presence of long chain fatty acids, triterpenes (friedelin and others), steroidal compounds (sitosterol, among others), diterpenes and sesquiterpenes. Caffeic acid derivatives can be found in PNE. Previous reports show that friedelin, sitosterol, some fatty acids and phenolic acids present cytotoxicity to several strains (Begin et al., 1988; Hoi et al., 2013; Matos et al., 2006; Ozcelik et al., 2011; Thao et al., 2010; Velasquez et al., 1993). Therefore, the presence of those compounds might explain the cytotoxicity observed.

Substances isolated from plants and fruits such as the aliphatic acetogenin constituents of avocado fruits show activity targeting EGFR/RAS/RAF/MEK/ERK1/2 pathway, which is considerably important in the carcinogenic process (D'Ambrosio et al., 2011). The lignin honokiol, isolated from magnolia species, induced stronger apoptotic activity in oral carcinoma cells when compared to 5-fluorouracil (Chen et al., 2011) and could be an alternative to chemotherapy treatments. Apoptotic death is desired in cancer treatment, given that necrosis is also associated with inflammation (Cho et al., 2011) and consequently induces more side effects in patients. In spite of the fact that these studies identified extracts and substances with high potential for cancer cytotoxicity and induction of apoptosis, none of them demonstrated specific activity against cancer cells.

## Conclusion

The current study showed that the hexane PNH fraction presented specific toxicity for tongue carcinoma cells (SCC-25). For these reasons, further studies to investigate the cell death profile induced by hexane PNH fraction (FHex) are necessary. Moreover, a thorough investigation on the constitution of the extracts and fractions, the isolation of compounds and the study of their activity on a molecular level are also required, although the results obtained until now show that it could be a great alternative for a more specific treatment for tongue cancer.

## Conflict of interests

The authors have not declared any conflict of interests.

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