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Isolation of flavonoids from *Tagetes patula* (French Marigold) flowers: Cytotoxic and oxidant activity in human cervical carcinoma cells

[Aislación de flavonoides de las flores de *Tagetes patula* (cempasúchil francés):
Actividad citotóxica y oxidante en células de carcinoma cervical humano]

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Abstract: *Tagetes patula*, known as French Marigold, belongs to the family Asteraceae. Human papillomavirus infection is considered one of the causes of cervical cancer. This study assessed the cytotoxic activity and intracellular oxidative capacity of compounds isolated from extract of *T. patula* flowers as anti-cancer cervical agents. Fraction F6 of n-butanol extract was subjected to column chromatography and HPLC-ESI-MS. The isolated compounds of *T. patula* were used to examine cytotoxic activity and the production of total reactive oxygen species in SiHa and HeLa cells; the cells were also characterized using scanning electron microscopy. Patulitrin was cytotoxic to SiHa and HeLa cells. An increase in ROS production was observed at different times of treatment of cells with patuletin and patulitrin. Scanning electron microscopy showed morphological changes in SiHa and HeLa cells. Thus, compounds isolated from *T. patula* have great treatment potential against cervical cancer.

Keywords: Asteraceae; Patuletin; Patulitrin; SiHa; HeLa

Resumen: *Tagetes patula*, conocida como cempasúchil francés, pertenece a la familia Asteraceae. La infección por el virus del papiloma humano se considera una de las causas del cáncer cervical. En este estudio, se evaluó la actividad citotóxica y la capacidad oxidativa intracelular de los compuestos aislados del extracto de las flores de *T. patula* como agentes anticancerígenos cervicales. La fracción F6 del extracto de n-butanol se sometió a cromatografía en columna y HPLC-ESI-MS. Los compuestos aislados de *T. patula* se utilizaron para examinar la actividad citotóxica y la producción total de especies reactivas de oxígeno en las células SiHa y HeLa; las células también se caracterizaron mediante microscopía electrónica de barrido. Patulitrina resultó citotóxica para las células SiHa y HeLa. Se observó un aumento en la producción de ROS en diferentes momentos del tratamiento de las células con patuletina y patulitrina. La microscopía electrónica de barrido mostró cambios morfológicos en las células SiHa y HeLa. Por lo tanto, los compuestos aislados de *T. patula* tienen un gran potencial de tratamiento contra el cáncer cervical.

Palabras clave: Asteraceae; Patuletina; Patulitrina; SiHa; HeLa

ABBREVIATIONS
CC: Column chromatography; CC_{50}: Cytotoxic concentration 50%; DMEM: Dulbecco's Modified Eagle's medium; DMSO: Dimethyl sulfoxide; HPLC-ESI-MS: High-Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry; HPV: *Human papillomavirus;* H_2O_2: Hydrogen peroxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide; NMR: Nuclear magnetic resonance; ROS: Oxigen-reactive species; SEM: Scanning electron microscopy.

INTRODUCTION
Cervical cancer is the third most common cancer among women (INCa, 2020). Human papillomavirus (HPV) infection is one of the most common sexually transmitted diseases worldwide (Hajdu *et al*., 2014; Kim & Kwak, 2015). National Institute of Cancer estimates that there are over 16.710 new cases and more than 6.526 deaths per year in Brazil (INCa, 2020). During the pre-clinical phase of cervical cancer (symptomless), precursor lesions (which precede disease onset) can be detected through preventive examination (Pap test). When diagnosed early, cervical cancer is 100% curable. As the disease progresses, symptoms such as vaginal bleeding, discharge, and pain appear (INCa, 2020).

The risk of pre-invasive and invasive cervical cancer lesions is strongly associated with persistent infection with HPV, particularly with type 16 (Mitteldorf, 2016). HPV type 18 accounts for approximately 10% of cases and other types for 5%. Cervical cancer risk factors are related to individual and viral characteristics and include exposure to HPV, viral oncogenicity, inefficiency of the patient's immune response, and presence of carcinogenic agents (Alexandre *et al*., 2020).

In Brazil, the prevalence of HPV is similar to that in the rest of the world, with 53.2% for HPV-16 and 15.8% for HPV-18 in the total population. Other types of cancer may be associated with HPV, including vaginal, vulvar, penile, anal, and oropharyngeal cancer (Ministerio de Saude, 2020). Platinum-based chemotherapeutic agents are effective in treating cervical cancer but cause a high number of serious side effects, including nephrotoxicity, ototoxicity, neurotoxicity, gastrointestinal reactions, and bone-marrow toxicity (Figueiredo *et al*., 2013; Zhao *et al*., 2016). Thus, new drug candidates with high efficacy and less side effects must be identified for the treatment of cervical cancer.

Despite the massive development of synthetic drugs, plants remain a major source of drugs (De San José *et al*., 2012). Recently, the number of studies on the biological activity of natural substances derived from medicinal plants increased considerably. The discovery of natural products with activity specific to some types of tumors would be useful in chemoprevention and cancer chemotherapy (Rahnama *et al*., 2017).

Many medicinal plants are rich in flavonoids, a group of complex molecules with anti-inflammatory, antioxidant, and potentially antitumor properties (Krzyzaniak *et al*., 2017). Patuletin and patulitrin are two of the main flavonoids found in *T. patula* flowers (Krzyzaniak *et al*., 2017). This plant is commonly known as French Marigold, belongs to the Asteraceae family, and is native to Mexico and Central America (Krzyzaniak *et al*., 2017).

Various biological activities have been reported in the literature for the isolated patuletin and patulitrin flavonoids including antioxidant activity (Munhoz *et al*., 2012; Yasukawa & Kasahara, 2013). The methanolic extracts of *T. patula* flowers and isolated patuletin were used to scavenge peroxide and superoxide radicals in chemical systems and human neutrophils, while simultaneously exerting cytotoxic and growth-inhibitory effects on human cancer-cell lines, in particular HeLa cells (Faizi *et al*., 2008; Kashif *et al*., 2015).

The biological activities of patuletin and patulitrin in SiHa and HeLa cells remain poorly studied despite their reported efficient cytotoxic and oxidant activities. Therefore, the present study aimed to isolate and identify the compounds present in an enriched fraction of *T. patula* flowers by reverse-phase column chromatography. Further, the study assessed the cytotoxic and oxidant activity of isolated patuletin and patulitrin in the above two cell lines, as well as in a human cervical carcinoma immortalized by HPV-16 and 18, and in an immortalized human keratinocyte line, HaCaT.

MATERIALS AND METHODS
Plant raw material
Tagetes patula seeds were purchased from Syngenta Flowers Brazil and organically grown at the Medicinal Plant Garden of the State University of Londrina (UEL), Londrina, Paraná (PR). *T. patula* flowers were collected in November 2011, under IBAMA-SISBIO license number 11995-6, authentication code 48926652, under the responsibility of J. C. P. Mello. A plant voucher is

deposited at the Herbarium of the State University of Maringá (Herbário under number 21.907, identified by Dr. Jimi Nakajima, Institute of Biology, Federal University of Uberlândia, Brazil). The flowers were dried in an oven at 38°C for 48 h. The plant material was milled with hammers (Tiger ASN-6).

Preparation of isolated compounds
Crude acetone extract (AE) was prepared at the Laboratory of Pharmaceutical Biology (PALAFITO) of the Department of Pharmacy of the State University of Maringá (UEM). The flowers, comminuted without separation by particle size, were degreased by dynamic maceration with n-hexane for 3 days. The solvent was exchanged for fresh solvent, as required, and then dried at room temperature. Later, the dried and degreased flowers were extracted with acetone (4% m/v) by using an Ultra-Turrax® (UTC115KT) for 5 min, and macerated for 15 h. The solution was subjected to turbo extraction for 20 min, at 5 min intervals, to ensure that the temperature did not exceed 40°C. Filtration was conducted under reduced pressure by using a Büchner funnel, and the residue was washed. The filtrate was concentrated under reduced pressure by using a rotary evaporator, with a maximum temperature of 40 °C, to eliminate all the organic solvent. The solution was frozen and freeze-dried (Christ®, Alpha 1-4) to obtain the crude AE.

       AE (105 g) was resuspended in 1 L of a methanol/water mixture (2:8, v/v) and subjected to liquid-liquid partitioning in five volumes of n-hexane (FH), dichloromethane (FD), ethyl acetate (FAE), and n-butanol (FB). The obtained fractions were concentrated by using a rotary evaporator under reduced pressure, and freeze-dried.

Reverse-phase column chromatography of subfraction F6 of n-butanol
FB (20 g) was subjected to column chromatography (CC) containing polyamide (CC6 Korngrobe, 0.05–0.16 mm; Macherey Nagel) in accordance with the method of Degani *et al.* (1998), with mobile phases of (%): H2O (100); MeOH (5-100); MeOH:AcOEt (9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9); acetone, in different volumes, collecting 10 mL/min per test tube. The fractions were pooled according to similarity in TLC (AcOEt:Formic acid: H2O; 90:5:5, v/v).

       Fraction F6 (250 mg, 9.19%) was again subjected to CC under the same conditions as before, with a mobile phase of 100% methanol. Subfraction

F6-5 (170 mg; 78.8%), after CC under the same conditions as before, yielded subfractions F6-5-3 (16 mg; 9.4%) and F6-5-4 (30 mg; 17.6%), which precipitated during the process of organic solvent removal and were analyzed by HPLC coupled to electrospray ionization MS (HPLC-ESI-MS) and NMR spectroscopy.

Identification of substances by mass spectrometry (HPLC-ESI-MS) and NMR
HPLC system used was Waters model 1525 μ coupled to a triple quadrupole mass spectrometer model Micromass Quattro micro™ equipped with electrospray ionization (ESI) interface. The software Mass Lynx™ version 4.0 (Waters) was used for data acquisition and processing. The mass spectrometer was operated under the following conditions: 2.5 kV capillary tension, 2 V extraction cone, 450ºC desolvation temperature, 30 V cone, 130ºC source temperature, 50 L/h cone gas flow, and 900 L/h desolvation-gas flow, using Argon as a collision gas (15-30 eV collision energy). Chromatography was performed using a reverse-phase column (Symmetry C18 3.5 µm; 75 × 4.6 mm, Waters, Milford, MA, USA) at room temperature. The mobile phase consisted of water with 0.1% v/v formic acid (solvent A) and ACN with 0.1% v/v formic acid (solvent B), under the following linear-gradient conditions: 0-1 min 5% B; 1-15 min 50% B. The flow of the mobile phase was 0.5 mL/min, and the injection volume of the sample dissolved in water-ACN 1:1, v/v was 10 µL. Initially, the precursor ions were selected in full-scan mode and then fragmented using the daughter-scan method to obtain the fragments (product ions). The fragments were identified by analyzing product ion data collected from the spectra in comparison with previously published data. The main peaks found in the chromatograms were identified as substances isolated from the subfractions of *T. patula*.

       The 1D and 2D NMR analyses were performed by using a Varian Mercury Plus 300 spectrometer (1H at 300 MHz and 13C at 75 MHz) for TMS and were shown to be the compounds patuletin (F6-5-3) and patulitrin (F6-5-4) by comparison with literature data. Both substances were used for cytotoxic and oxidative assays and for SEM.

Cell lines
The immortalized human cervical carcinoma cell lines HPV-18 and HPV-16 and the immortalized human-skin keratinocyte line HaCaT were used in

this study. Plastic tissue-culture flasks with vented screw caps (TPP®) were used to culture the cells in DMEM (GIBCO®) supplemented with 10% FBS (GIBCO®) and antibiotics (100 µg/mL streptomycin and 100 µg/mL penicillin) in humid incubators at 37°C with 5% CO_2 (Fischer Scientific®). For maintenance, the cells were observed daily under an inverted microscope (Olympus®, model CKX 41). Periodically, sub-cultures were prepared after monolayer (80-90% confluence) formation.

Assessment of the cytotoxic activity of compounds isolated from T. patula

Cell viability was assessed using the MTT assay. SiHa and HeLa (2.5×105 cells/mL) and HaCaT (5.0×105 cells/mL) cells were seeded in 96-well microtiter microplates with DMEM supplemented with 10% FBS, and then kept in an incubator at 37ºC with 5% CO_2 for 24 h until the formation of a cell monolayer.

The isolated substances were diluted in DMSO. Samples were solubilized using an ultrasound bath for 15 min. Subsequently, the cells were treated with different concentrations (100, 50, 25, 10, 5, and 1 µg/mL) of compounds isolated from *T. patula* (patuletin and patulitrin) for 24 and 48 h under the aforementioned conditions. After treatment, the cells were washed with 100 µL PBS and 2 mg/mL MTT was added subsequently. Culture plate was incubated for 4 h in an incubator at 37ºC protected from light. Formazan crystals formed in wells were solubilized in DMSO and absorbance was read at 570 nm in a microplate spectrophotometer (Bio Tek – Power Wave XS). The percentage of viable cells was calculated in relation to the control. CC_{50} (50% cytotoxic concentration) was determined by non-linear regression analysis. The concentrations used in other experiments were determined based on the CC_{50}.

Total ROS production

SiHa, HeLa (2.5×105/mL), and HaCaT (5.0×105/mL) cells were seeded in a 96-well black microtiter plate; some of the cells were treated with the test compounds isolated from *T. patula* at CC_{50} (SiHa: 90.64 µg/mL patuletin, 7.41 µg/mL patulitrin; HeLa: 55.26 µg/mL patuletin, 11.07 µg/mL patulitrin; HaCaT: 103.89 µg/mL patuletin, 59.37 µg/mL patulitrin) diluted in DMEM and incubated

for 24 h and then washed in PBS. Subsequently, 5 µM H2DCF-DA (2′,7′-dichlorofluorescein diacetate) was added and the plate was incubated for 45 min at 37ºC in the dark. Fluorescence was quantified using a spectrofluorimeter (VICTOR™ X3, PerkinElmer) at 488/530 nm excitation/emission wavelengths. Hydrogen peroxide (100 mM) was used as a positive control.

Scanning Electron Microscopy

SiHa, HeLa, and HaCaT cells were seeded in 24-well plates with chip coverslips, at a density of 2.5×105 cells/well (SiHa and HeLa) or 5.0×105 cells/well (HaCaT) and incubated for 24 h in an incubator at 37ºC with 5% CO_2. After 24 h, the cells were treated with CC_{50} of the compounds isolated from *T. patula* (SiHa: 90.64 µg/mL patuletin, 7.41 µg/mL patulitrin; HeLa: 55.26 µg/mL patuletin, 11.07 µg/mL patulitrin; HaCaT: 103.89 µg/mL patuletin, 59.37 µg/mL patulitrin) diluted in DMEM, or only with DMEM for the cell control, and then incubated for 24 h. On the third day, the cells were prefixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, and dehydrated in 50, 70, 80, 90, 95, and 100% ethanol (15 min in each and 3 times in 100% ethanol). After the critical point and gold metallization, external differences in cell morphology were observed under the Scanning Electron Microscope FEI QUANTA 250.

Statistical analysis

Data shown in tables and graphs express the mean ± standard deviation of at least three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey post-test, considering $p < 0.05$ as significant. All statistical tests were performed using the software Statistica 8.0.

RESULTS
Identification of the chemical compounds

The chemical compounds were characterized by HPLC-ESI-MS in the positive ionization mode ([M+H]+). The chemical compounds present in *T. patula* flowers were identified by comparing the product ions with those described in the literature.
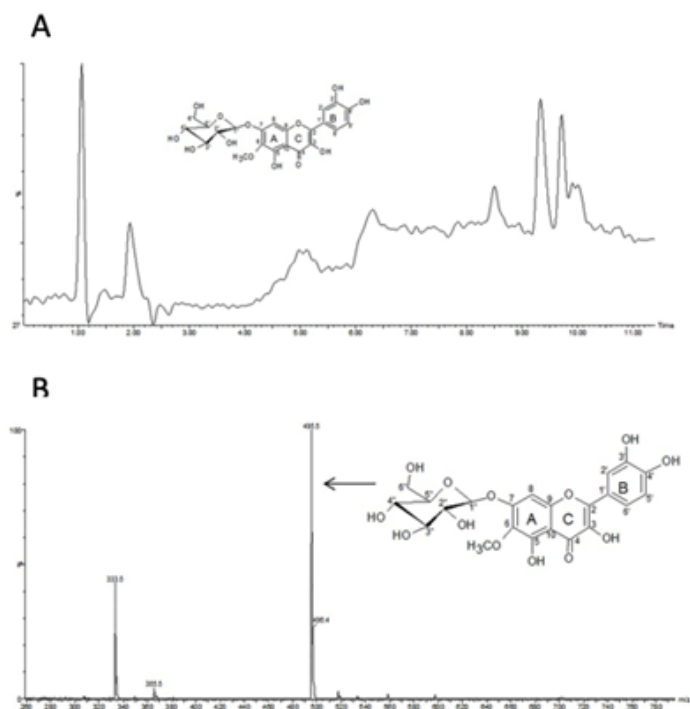
The HPLC-ESI-MS showed a major substance (F6-5-3) at retention time tr: 8 min, isolated (Figure No. 1) in the sample.

Figure No. 1
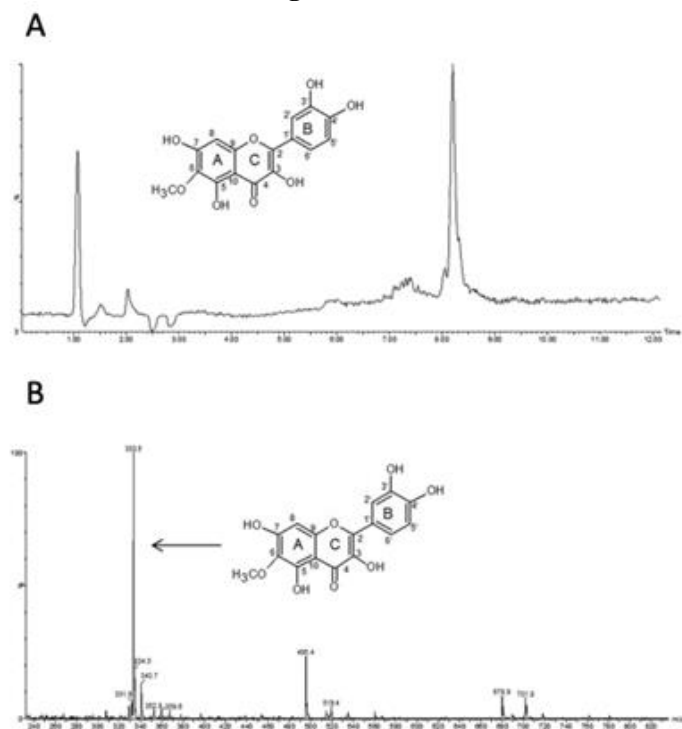
(a) ESI chromatographic profile of F6-5-3 of *T. patula L*. (b) ESI mass spectrum of F6-5-3 of *T. patula* L.
(Peak: PM: 333.5; positive mode, corresponding to patuletin)

Figure No. 2

(a) ESI chromatographic profile of F6-5-4 of T. patula (b) ESI mass spectrum of F6-5-4 of T. patula
(Peak: PM: 495.5; positive mode, corresponding to patulitrin)

Moreover, a major substance (F6-5-4) was observed at retention times tr: 8 min and tr: 9.30 min, respectively, in the sample (Figure No. 2).

Cytotoxic activity of the compounds isolated from T. patula

MTT (3-(4,5-dimethylthiazol-2-yl)-2.5-diphenylte-trazolium bromide) assay was performed in SiHa, HeLa, and HaCaT cells exposed to different concentrations (100, 50, 25, 10, 5, and 1 µg/mL) of patuletin and patulitrin for 24 and 48 h. When the cells were exposed for 24 h to the compounds, patulitrin was cytotoxic only to SiHa and HeLa cells with a maximum CC_{50} of 14.57 and 13.96 µg/mL, respectively. Conversely, patuletin was not cytotoxic to SiHa, HeLa, or HaCaT cells, and the maximum CC_{50} found were 102.68, 60.46, and 109.57 µg/mL, respectively (Table No. 1).

Table No. 1
Cytotoxic activity of compounds isolated from *T. patula* in SiHa, HeLa, and HaCaT cell lines (CC_{50}) (mean ± standard deviation of three experiments performed in triplicate) after 24 h and 48 h of treatment.

Samples / Cells	SiHa		HeLa		HaCaT	
	24 h	48 h	24 h	48 h	24 h	48 h
Patuletin	90.6±10.5	49.6±10.4	55.3 ± 4.6	42.5±12.4	103.9 ± 5.1	62.9±10.6
Patulitrin	7.4±6.1	13.3±0.6	11.1 ± 4.1	23.6±3.6	59.4 ± 3.7	110.8±2.4

CC_{50} µg/mL = 50% cytotoxic concentration in viable cells. SD = standard deviation

Conversely, when the cells were exposed to the compounds for 48 h, patulitrin was cytotoxic to SiHa and HeLa cells. Under this condition, the maximum CC_{50} were 13.68 and 26.16 µg/mL, respectively. In contrast, patuletin showed no cytotoxicity at a maximum CC_{50} of 59.93 µg/mL to SiHa cells and 56.20 µg/mL to HeLa cells. The compounds showed no toxicity to control HaCaT cells at a CC_{50} of 72.99 µg/mL for patuletin and 113.14 µg/mL for patulitrin (Table No. 1).

Total ROS production

SiHa, HeLa, and HaCaT cells were treated with 90.6, 55.3, and 103.9 µg/mL patuletin and 7.4, 11.1, and 59.4 µg/mL patulitrin. These compounds increased ($p<0.001$) ROS production in SiHa and HeLa cells.

In SiHa cells, patulitrin showed a fluorescence intensity of 153,700 at 24 h of exposure; in comparison, the positive control with hydrogen peroxide showed an intensity of 16,170 at 24 h (Figure No. 3a). In HeLa cells, the intensity was 73,700 for patulitrin and 76,620 for the positive control at 3 h (Figure No. 3b). The DMSO control (990-µL DMEM + 10-µL DMSO) showed a fluorescence intensity of 27,340 at 3 h of treatment; in SiHa cells treated with patulitrin (Figure No. 3a), the fluorescence intensity was 70,640. In HeLa cells (Figure No. 3b), DMSO had no effect, with fluorescence intensities of 5,825, 15,870, 20,510, 25,840, and 53,270 at 0, 1, 2, 3, and 24 h, respectively.

In HaCaT cells (Figure No. 3c), no difference in fluorescence intensity was found when comparing negative control cells with the DMSO control, with 1,543, 4,573, 6,514, 9,755, 28,020, and 1,552, 3,779, 4,475, 10,430, and 30,770 at 0, 1, 2, 3, and 24 h, respectively. The comparison of the positive control with the isolated compounds showed that the fluorescence intensity of the patuletin at 3 and 24 h was 43,290 and 35,780, respectively. The fluorescence intensity of the patulitrin compound at 3 h was 52,180.
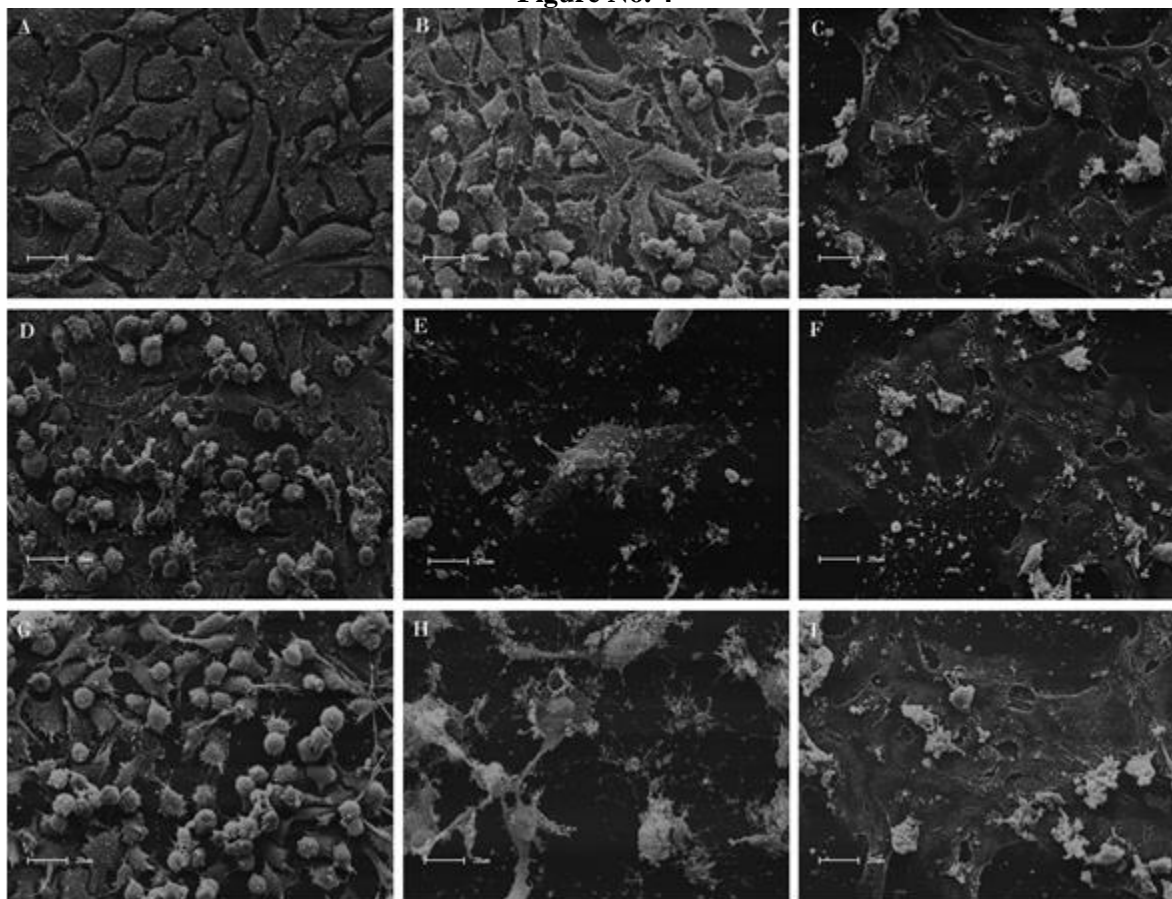
Scanning electron microscopy (SEM)

SEM highlights the surface of SiHa, HeLa, and HaCaT cells. SiHa, HeLa, and HaCaT cells without treatment had an intact cell monolayer and with normal morphology and size (Figure No. 4A, 4B, and 4C). After treating SiHa cells with patuletin and patulitrin, the cell monolayer was damaged; these compounds caused cell death, leading to the detachment of the cells from the monolayer (Figure No. 4D and 4G). Treatment of HeLa cells with patuletin and patulitrin showed damaged cell monolayer and altered cell size (Figure No. 4E and 4H). HaCaT cells treated with patuletin (F) and patulitrin (I) showed no changes.

Figure No. 3
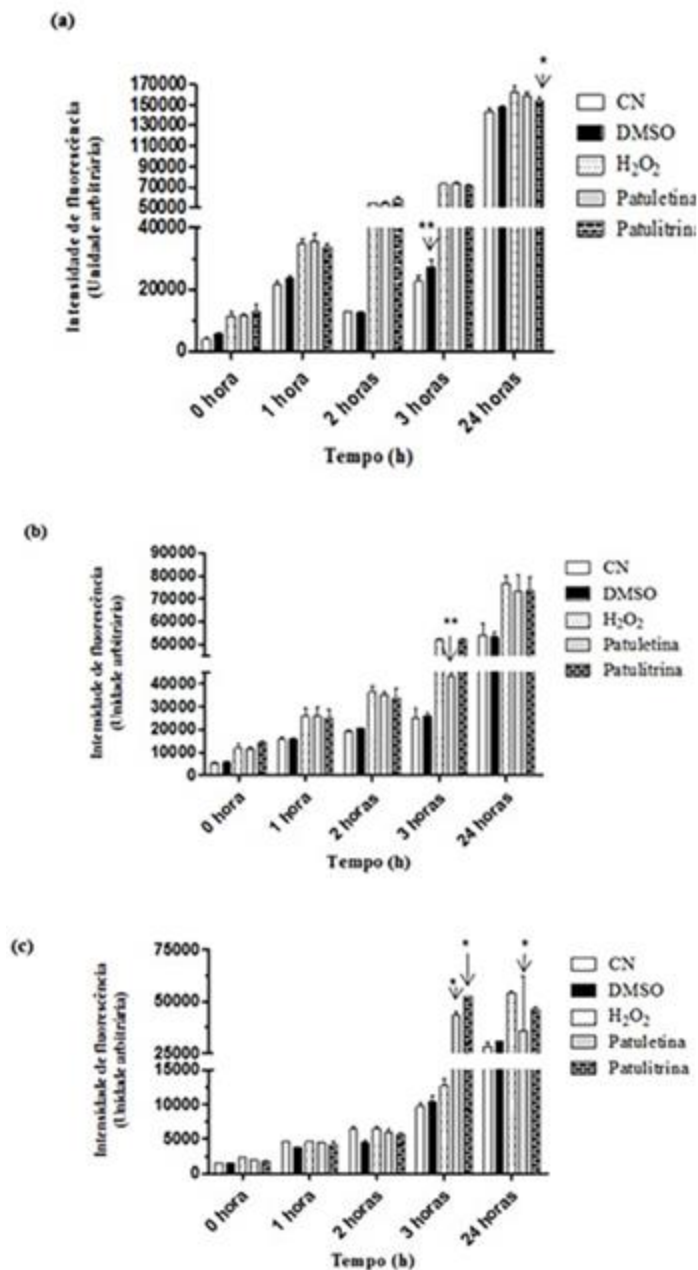
(a) **Spectrofluorimetric assessment of reactive oxygen species (ROS) production in SiHa cells treated with patuletin and patulitrin. (b) Spectrofluorimetric assessment of ROS production in HeLa cells treated with patuletin and patulitrin. (c) Spectrofluorimetric assessment of ROS production in HaCaT cells treated with patuletin and patulitrin. Untreated cells were used as the negative control, and 30% hydrogen peroxide (100 mM) was used as the positive control. Data are expressed as the mean fluorescence percentage ± SD of at least three independent experiments performed in triplicate. The asterisks indicate a significant difference relative to the control group (one-way ANOVA, Tukey post-test, *$p<0.001$; **$p<0.01$).**

Figure No. 4

Morphological aspect of the cells observed under scanning electron microscope. Image A: control SiHa cells (without treatment). Image B: control HeLa cells. Image C: control HaCaT cells. Images D, E, F: SiHa, HeLa and HaCaT cells treated with patuletin. G, H, I:SiHa, HeLa and HaCaT cells treated with patulitrin

DISCUSSION

To isolate the substances from n-butanol semi-purified fraction, three solvents were used: ethyl acetate (AcOEt), methanol (MeOH), and distilled water. According to Leitão (2005), elution systems with four and sometimes five solvents are common. Studies have shown that elution systems containing water, methanol, ethyl acetate, and n-hexane work well for the separation of natural compounds and are commonly used for this purpose (Pauli *et al*., 2008).

HPLC is a technique often used to isolate phenols, such as phenolic acids, flavonoids, procyanidins, and anthocyanins (Stefova *et al*., 2003; Angelo & Jorge, 2007). The reverse-phase column C-18 was used in this study. According to the literature, this type of column is ideal to use for the separation of different classes of phenolic compounds (Tarnawski *et al*., 2006; Angelo & Jorge, 2007;

Longhini *et al*., 2013). The reverse-phase HPLC system (RP-HPLC) consists of a stationary phase of lower polarity and of a mobile phase of higher polarity, primarily using water-based solutions (Kazakevich & Lobrutto, 2007). The main advantages of reverse-phase columns are rapid establishment of column equilibrium after mobile phase change, ease of use of gradient elution, faster analysis, and good reproducibility of retention times. Reverse phase is often used to separate solutes of different polarities, molar masses, and chemical functionalities (Tonhi *et al*., 2002).

The mobile phase used in our study was a gradient system of water: ACN acidified with trifluoroacetic acid. According to the literature, most analyses for flavonoids using a C-18 reverse-phase column use methanol: water or ACN: water as the mobile phase. The mobile phase is sometimes

acidified with formic acid, acetic acid, and/or phosphoric acid to aid in mass analyses (Stefova *et al*., 2003).

The F6-5-3 and F6-5-4 subfractions were analyzed by HPLC-IES-MS/MS and the data were compared with the literature (Parejo *et al*., 2004). In the mass spectral data, through the fragmentation of the precursor ion corresponding to F6-5-3 ([M-H]-) m/z 333 and F6-5-4 ([M-H]-) m/z 495, substances corresponding to patuletin and patuletin-7-O-β-glucose were suggested, respectively. In addition, these substances were compared by HPLC with data of Krzyzaniak *et al*. (2017), that were presented as the major component with the retention time of 8 min.

Few studies have been performed thus far on the biological activity of patuletin, quercetagetin, and their glycosidic derivatives that are typical flavonoids of the genus of *T. patula* (Krzyzaniak *et al*., 2017). While the number of published studies on the biological activity of extracts, fractions, and their isolates of *T. patula* increased in recent years, no data on the possible antitumor activity of extracts, fractions, and isolates of *T. patula* are available in the literature.

Cisplatin was considered for a long time as the most active drug for treating cancer of uterine cervix (Thigpan *et al*., 1981). Currently, chemotherapy combination is more commonly used now as it elicits a better response. Cisplatin/paclitaxel combination is one of the first line chemotherapy considered in the treatment of cervical cancer (Monk *et al*., 2009; Markman, 2014). The initial chemotherapy typically gives a satisfactory therapeutic effect. However, the chemoresistance gradually develops after several months (Al-Dimassi *et al*., 2014), and has become a main limitation to this cancer treatment (Al-Dimassi *et al*., 2014).

Studie conducted with vinca alkaloids (vincristine, vindesine, and vinblastine) from *Lochnera rosea* (L.) Rchb. ex K. Schum., commonly known as *Vinca rosea*. *Taxus brevifolia*, a plant from which taxol is extracted, demonstrated high antitumor activity in the treatment of breast, ovarian, and lung cancers (Abreu *et al*., 2017).

Silva *et al*. (2012), Santos *et al*. (2012), Povh & Alves (2013), and Oberlies *et al*. (1997), reported that the extract of soursop (*Annona muricate*) leaves exhibited cancer-cell proliferation.

While the toxicity of medicinal plants may seem "simpler" than that of conventional pharmaceutical drugs, it is a public health concern (Alves *et al*., 2007)[34]. Although natural products have a broad-spectrum therapeutic activity and a low incidence of side effects, their toxic levels are not considered innocuous. Accordingly, toxicological tests must be performed to provide scientific data on medicinal plant safety and toxicity in humans (Assemi, 2001).

In vitro cytotoxicity tests are important for assessing the toxicity of new compounds at the initial stages of development of pharmaceutical drugs (Mello & Petrovick, 2000); the balance between the pharmacological and toxicological effects of a compound is a key requirement for its applicability as a future therapeutic agent (Mello & Petrovick, 2000).

The search for substances with cytotoxic and potentially anti-cancer activity has always been one of the priorities of medicinal chemistry. A large number of different approaches have been used in this search; however, the discovery of selective antitumor substances still remains a goal of cancer research (Navarini *et al*., 2009).

The results from the MTT assay in the study by Zhao *et al*. (2016), showed that HeLa and SiHa cells proliferation was significantly inhibited by dioscin in a time- and dose-dependent manner, particularly in HeLa cells. In comparison with the control groups, cell proliferation inhibition rates after treatment with 5.0 µg/mL dioscin for 12 and 24 h were 47.79% and 80%, respectively, whereas the cell proliferation inhibition rate in SiHa cells treated with 5.0 µg/mL dioscin for 24 h was 53.62%.

Apoptotic cell death can be induced by increasing the intracellular levels of ROS, especially H_2O_2. Flavonoids are known to increase the levels of ROS in various tumor cell lines (Guzy *et al*., 2010). Reversible oxidative modifications and oxidative damage differ only in the intensity with which the phenomena triggered by ROS occurs. Oxidative damage occurs when oxidative changes occur at a high intensity and are sustained over time, thereby surpassing the cellular antioxidant capacity. This leads to uncontrolled and sometimes lethal functional and structural changes in the cells. This condition characterizes free-radical intoxication commonly known as oxidative stress (Cardoso *et al*., 2006). Oxidative stress is a consequence of an imbalance in the quantity of total ROS, commonly known as free radicals. This imbalance can be caused by several factors related to the increase in the production of ROS and/or to the decrease in antioxidant availability (Cardoso *et al*., 2006; Andrade *et al*., 2010). Furthermore, oxidative stress induces

lipoperoxidation, specifically through lipid hydroperoxides that affect lipid-containing structures (Andrade *et al*., 2010; Kudryavtseva *et al*., 2016).

The production of reactive oxygen species by patuletin and patulitrin in SiHa, HeLa, and HaCaT cells was determined in an assay with H2DCF-DA, a cell-permeable non-fluorescent dye oxidized by intracellular ROS to fluorescent 2′7′-dichloroflurescein (DCF) (Halliwell & Whiteman, 2004).

Recently, studies conducted by Zhao *et al*. (2016) showed that the anti-cancer effects of ROS might affect the mitochondrial-membrane potential and membrane permeability. Simultaneously, the excess of ROS may lead to lipid peroxidation, protein oxidation, enzyme inactivation, and oxidative DNA damage. Thus, ROS is considered a key target for the treatment of cervical carcinoma.

Marcondes *et al*. (2015), investigated MCF-7 cell changes after treatment with clotrimazole (an anti-fungal medication) by SEM. The results showed a large number of vesicles on the cell surface, typical of cancer cells because they are crucial for cell signaling. After treatment with micellar clotrimazole for 24 h, changes in cell shape were observed under SEM, clearly showing the loss of adhesion. Cells with a round shape suggest the beginning of a process of cell death evidenced by decrease in the number of cells observed and in cell viability.

A study conducted by Martínez *et al*. (2017), also noted morphological changes in human tracheal carcinoma (Hep-2 cell line) after photodynamic therapy using the ZnPc photosensitizing agent, thus suggesting that Hep-2 cells undergo cell death. Patuletin and patulitrin isolated from *T. patula* flowers show cytotoxic and oxidant potential against the human cervical carcinoma cells SiHa and HeLa. The current knowledge of patuletin and patulitrin properties indicates a promising future for their use as agents to treat cervical cancer.

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