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Original Article

Antiproliferative potential of solidagenone isolated of *Solidago chilensis*

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ABSTRACT

Plants are considered among the main sources of biologically active chemicals. The species *Solidago chilensis* Meyen, Asteraceae, is native to the southern parts of South America, where the aerial parts of the plant are commonly used for the treatment of inflammatory conditions. However, the effects of *S. chilensis* on human cancer cells remain to be elucidated. In this study, we evaluated the antiproliferative effects of the hydroalcoholic and dichloromethane extracts of *S. chilensis*, as well as their chemical constituents quercitrin and solidagenone against the five human tumor cell lines *in vitro*. The dichloromethane extract showed a promisor antiproliferative effects *in vitro*, especially against glioma cell line. Besides, the hydroalcoholic extract and quercitrin were inactive. The diterpene solidagenone showed highly potent antiproliferative effects against breast (MCF-7), kidney (786-0), and prostate cancer (PC-3) cells (total growth inhibition: TGI < 6.25 μg/ml). Solidagenone meets the theoretical physico-chemical criteria for bioavailability of drugs, according to the "Rule of Five" and, by theorical studies, the observed biological effects were probably related to the interaction of the molecule with nuclear receptors and as an enzymatic inhibitor. This study contributes to chemical study and to the identification of antiproliferative molecules in *S. chilensis*.

Introduction

Cancer is one of the leading causes of unnatural death globally, and is considered a major public health problem (Siegel et al., 2016; Wang et al., 2016). Cancer therapy is based primarily on the association of surgical resection of tumors with radiotherapy, immunotherapy and/or chemotherapy (Kakde et al., 2011; Baskar et al., 2012). However, many cancers still present modest responses to clinical protocols, limiting the indication and efficacy of treatment of both primary tumors and metastases (Costa-Lotufo et al., 2010). The efficacy of cancer drugs is often limited by their insolubility and instability, the low rate at which the tissue absorbs them, and drug resistance of the tumor (Akindele et al., 2015). In addition, many antineoplastic agents have high rates of adverse reactions and toxicity (Prakrash et al., 2013).

All these drawbacks presently associated with available chemotherapeutic agents provide the impetus for the search of newer, more efficacious, and better tolerated drugs. In order to achieve more effective and safer results, pharmacological studies with substances extracted from plants as well as synthetic derivatives from these natural compounds have been intensified (Chorawala et al., 2012; Newman and Cragg, 2016). Medicinal

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plants have been a valuable source of successful therapeutic agents, the production of chemically diversified secondary metabolites are optimized to exert biological functions (Atanasov et al., 2015). Natural products are a major source of effective drugs for cancer treatment and often inspire the development of new potential agents (Newman and Cragg, 2012).

The Asteraceae family has many species with ethnopharmacological applications, including anti-inflammatory, antiseptic, and anti-tumor properties. Phytochemical studies of Asteraceae species report the presence of triterpenes, sesquiterpene lactones, lignans, flavonoids, and caffeoylquinic acid derivatives (Costa et al., 2015).

In South America, the species *Solidago chilensis* Meyen, Asteraceae, is known as arnica-brasileira, arnica-do-campo, erva-lanceta, and espiga-de-ouro (Lorenzi and Matos, 2002). The aerial parts of the plant are commonly used for their diuretic potential, analgesic abilities, anti-inflammatory, anti-rheumatic and healing effects, as well as for the treatment of burns (Lorenzi and Matos, 2002; Assini et al., 2013).

Chemical studies have shown that the ethanolic extract of the aerial parts of *S. chilensis* contains flavonoids (quercitrin, quercetin and rutin), diterpenes (solidagenone), along with, α-epinasterylglycopyranoside, caffeic acids, chlorogenic, clerodanic, β-farnesene, α-amirin, and other derivatives of solidagolactol (Torres et al., 1987; Schmeda-Hirshmann, 1988; Torres et al., 1989). Solidagenone is the major chemical compound of the dichloromethane extract of plant inflorescences, and quality parameters was proposed by high performance liquid chromatography (Valverde-Soares et al., 2009).

Preclinical studies have shown that the aqueous extract of the inflorescence had wound-healing (Facury-Neto et al., 2004) and gastroprotective effects (Rodríguez et al., 2005; Bucciarelli et al., 2010). The hydroalcoholic extract of the aerial parts have been shown to have anti-inflammatory effects (Tamura et al., 2009; Silva et al., 2010), and was recently found to have hypolipidemic (Roman Junior et al., 2015) and hypoglycemic activities (Schneider et al., 2015). However, there are no reports evaluating the antiproliferative effects of the plant.

The present study aimed to produce extracts of different polarities from *S. chilensis*, isolate their chemical constituents, and evaluate their antitumor effects *in vitro*.

Materials and methods

Solvents and chemicals

All solvents and reagents used were of analytical grade, and distilled and deionized water was used. The other solvents used were methanol, ethanol, acetonitrile, ethyl acetate, dichloromethane, chloroform and acetic acid (Vetec®, Rio de Janeiro, Brazil). Quercitrin and solidagenone was obtained from Sigma-Aldrich®, St. Louis, MO, USA. Chromatographic analysis was performed using HPLC with a Varian® Pro-Star chromatograph with an automatic injector (20 μl handle), ternary pump gradient, UV/Vis detector, and Kromasil® ODS column (5 mm) C-18 reverse phase (250 × 4.5 mm). HRMS were run in a QSTAR XL Q-TOF (Applied Biosystems) using electrospray ionization (ESI) with an Agilent 1100 HPLC. The NMR (^{1}H and ^{13}C) experiments were performed on a Bruker Avance 400 (400 and 100 MHz, respectively) spectrometer with the substances diluted in $CDCl_3$ and CD_3OD. The ^{1}H and ^{13}C NMR chemical shifts were expressed in ppm (δ) using TMS (0.00 ppm) as an internal reference and the coupling constants (J) in Hz.

Plant material

The aerial parts of *Solidago chilensis* Meyen, Asteraceae, were collected in Chapecó (SC), Brazil (26°59′31.03″ S and 52°41′17.89″ O), in February of 2016. The plant material was identified by Osmar dos Santos Ribas, curator of the Municipal Botanical Museum of Curitiba (PR), where a voucher specimen was deposited (MBM #356792).

Production of extracts

The aerial parts were reduced to small fragments and subjected to drying (25 °C), protected from direct light and humidity. The dehydrated plant species were ground in a knife mill (Ciemlab®, CE430) and sieved to select particles of 425 μm (35 Tyler/Mesh).

Aliquots of dry milled *S. chilensis* aerial parts (10 g) were extracted succecively with dichloromethane (200 ml) and ethanol (70%, 200 ml) by maceration during 5 days. After filtration, the solvents were eliminated under reduced pressure using a rotary evaporator and the resulting dichloromethane (DES) and hydroalcoholic (HES) extracts from *S. chilensis* were lyophilized, weighed, and stored in a freezer at − 20 °C.

HPLC analysis of extracts from Solidago chilensis

Chromatographic analysis by HPLC of the HES was carried out using Kromasil® ODS column (5 μm), RP-18 reverse phase (250 × 4.5 mm) at a temperature of 24 ± 2 °C. Two solvent systems was used: H_2O:AcOH (40:1 v/v; solvent A) and CH_3CN (solvent B) at a flow rate of 1 ml/min. The gradient used was 86% A for 15 min, 65% A for 30 min, and 100% B for 2 min. Detection was performed at 360 nm, and the results were compared with an authentic external standard, followed by UV/Vis spectrometry (Apáti et al., 2006).

HPLC analysis of the DES was performed using Kromasil® ODS column (5 μm), RP-18 reverse phase (250 × 4.5 mm) at a temperature of 25 ± 2 °C. As mobile phase was used an isocratic solvent system consisting of H_2O (30%; solvent A) and MeOH (70%; solvent B) for 15 min, with a flow rate of 1 ml/min. Detection was performed at 220 nm, and the results were compared withan authentic external standard (solidagenone), followed by UV/Vis spectrometry (Valverde-Soares et al., 2009).

Chemical isolation

An aliquot of HES (20 g) was diluted with water (200 ml) followed by mechanical agitation (20 min). Subsequently, the resulting solution was transferred to a separating funnel and submitted to liquid/liquid partition successively with hexane and EtOAc (ten times per solvent, 500 ml each). After solvent evaporation, an aliquot (2 g) of the EtOAc fraction was dissolved in $CHCl_3$ (minimal amount) and submitted to liquid column chromatography using silica gel (0.063–0.200 mm; Merck®, Darmstadt, Germany) as the stationary phase and eluents $CHCl_3$ and EtOH (80:20 v/v) in increasing polarity up to 80% EtOH (v/v) as mobile phase. The eight subfractions obtained were similarly pooled by using thin layer chromatography (TLC) with EtOAc:MeOH:H_2O (100:13.5:10 v/v) as the mobile phase and analyzed using a UV/Vis spectrometer at 366 nm and revealed with H_2SO_4 (10% in methanol) followed by heating at 110 °C (10 min). Subfraction 6 (0.06 g) was observed as a spot by the TLC analysis, yielding the compound **1**.

A sample of DES (3 g) was dissolved in a sufficient quantity of hexane and subjected to liquid column chromatography using silica gel (Merck®, Darmstadt, Germany) as the stationary phase and a solution of hexane and EtOAc (90:10 v/v) in increasing polarity to 90% (v/v) of EtOAc for elution. Ten subfractions were obtained,

which were pooled by means of TLC using hexane:EtOAc (80:20 v/v) as the mobile phase with further analysis in a UV/Vis spectrometer at 366 nm, and revealed with H_2SO_4 (10% in methanol) followed by heating at 110 °C (10 min). The subfraction 9 (0.04 g) showed only spot yielding the compound **2**.

Antiproliferative assay

The antiproliferative effects of HES, DES, and compounds **1** and **2** were investigated using the protocol described by Monks et al. (1991). The assays were performed using a panel with five human cancer cell lines [glioblastoma (U-251), breast (MCF-7), kidney (786-0), non-small cell lung (NCI-H460) and prostate (PC-3) cells] kindly provided by Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MA, USA. Stock and experimental cultures were grown in 5 ml of complete medium [RPMI-1640 supplemented with 5% fetal bovine serum and 1% penicillin:streptomycin mixture (1000 U/ml:1000 mg/ml)]. Stock solutions each sample (5 mg) were prepared in DMSO (50 μl) followed by successive dilutions in complete medium affording final concentrations of 0.25, 2.5, 25, and 250 μg/ml. Doxorubicin was used as a positive control at final concentrations of 0.025, 0.25, 2.5, and 25 μg/ml. Cells in 96-well plates (100 μl cells/well, cell densities: 3–7 × 10^4 cells/ml) were incubated with each of the four concentrations of the sample solution or doxorubicin (100 μl/well) in triplicate, for 48 h at 37 °C and 5% CO_2. Before (T0 plates) and after (T1 plates) sample addition, the cells were fixed with 50% trichloroacetic acid (50 μl/well) and stained with sulforhodamine B (50 μl/well) to quantitate cell proliferation using the reading at 540 nm. The TGI (sample concentration that resulted in total cellular growth inhibition) values were determined through non-linear regression applied to a sigmoidal curve using Origin 8.0 software (OriginLab Corporation).

Calculation of molecular properties

The molecular properties were calculated on basis of simple molecular descriptors used by "Lipinski's rule of five" (Lipinski, 2004), to estimate the oral bioavailable. The five properties consist of molecular weight (u), hydrogen donor; acceptors, Log *P*, and total polar surface area (PSA; Å^2) which were calculated using the online chemoinformatics tool molinspiration (2018).

Results

HPLC analysis

The HES chromatogram (360 nm) showed great similarity with the quercitrin standard (RT = 9.82 min), indicating that this compound may be the major constituent of the extract. The DES chromatogram (220 nm) revealed that solidagenone (RT = 4.23 min) was the constituent present in greater concentration (Fig. 1).

Chemical compounds of S. chilensis

By chromatographic fractionation, compounds **1** and **2** were isolated from HES and DES, respectively. These compounds were identified by comparison of their experimental spectra (^{1}H NMR, ^{13}C NMR and ESI-MS) with those previously described: quercitrin (**1**) (Tiberti et al., 2007;Correia et al., 2008) and solidagenone (**2**) (Rodríguez et al., 2005).

Quercetin-3-*O*-rhamnoside (quercitrin; **1**): This compound was obtained as a yellow crystalline powder, with a melting point of 183–185 °C (water): $C_{21}H_{20}O_{11}$; ESI-MS: 449.0 $[M+H]^+$; ^{1}H NMR (400 MHz, CD_3OD, δ, ppm): 0.94 (3H, d; *J* = 6.1 Hz; 6″-H), 3.35 (1H, dd; *J* = 9.4: 9.4 Hz; 4″-H), 3.43 (1H, dq; *J* = 9.4: 6.1 Hz; 5″-H), 4.23 (1H, dd; *J* = 3.4: 1.5 Hz; 2″-H), 4.76 (1H, dd; *J* = 9.4: 3.4 Hz; 3″-H), 5.35 (1H, d; *J* = 1.5 Hz; 1″-H), 6.20 (1H, d; *J* = 2.1 Hz, 6-H), 6.37 (1H, d; *J* = 2.1 Hz, 8-H), 6.91 (1H, d; *J* = 8.3 Hz; 5′-H), 7.31 (1H, dd; *J* = 8.3: 2.1 Hz; 6′-H), 7.34 (1H, d, *J* = 2.1 Hz; 2′-H); ^{13}C NMR (100 MHz, CD_3OD, δ, ppm): 17.8 (C-6″-CH_3), 72.0 (C-2″), 72.2 (C-3″), 72.2 (C-4″), 73.4 (C-5″), 94.9 (C-8), 100.0 (C-6), 103.7 (C-1″), 106.4 (C-10), 116.5 (C-5′), 117.1 (C-2′), 123.0 (C-6′), 123.1 (C-1′), 136.4 (C-3), 146.5 (C-3′), 150.0 (C-2), 158.7 (C-9), 159.4 (C-4′), 163.4 (C-5), 166.0 (C-7), 179.8 (C-4).

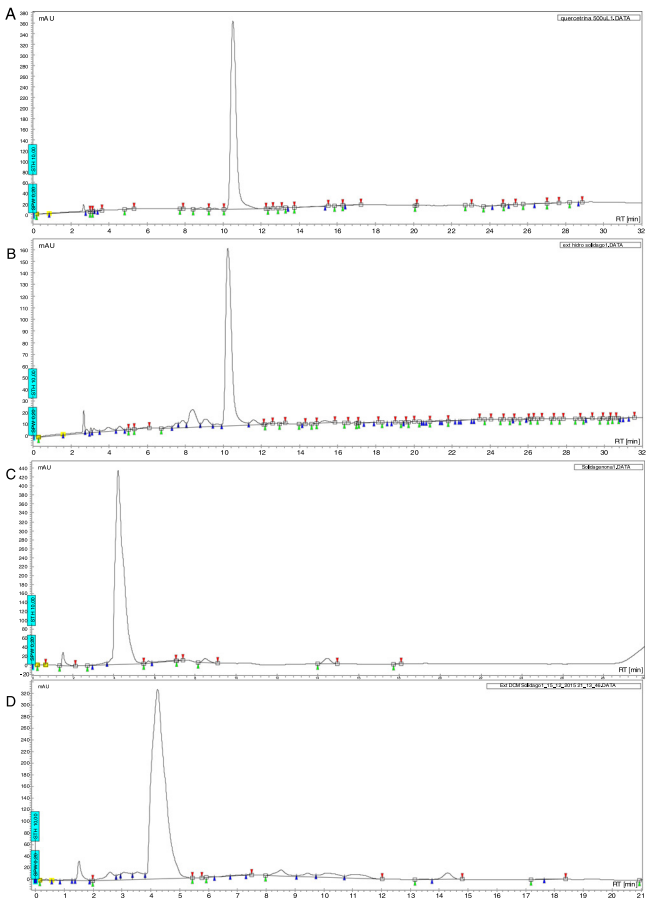
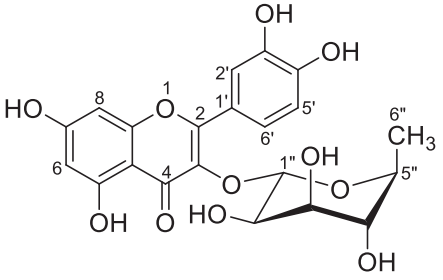


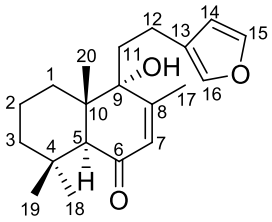
Fig. 1. HPLC chromatographic profile of *Solidago chilensis* aerial parts: (A) quercitrin (RT: 9.82 min); (B) hydroalcoholic extract; (C) solidagenone (R_f 4.12 min); (D) dichloromethane extract.



1

(4R,4aS)-4-[2-(furan-3-yl)ethyl]-4-hydroxy-3,4a,8,8-tetramethyl-1,4,4a,5,6,7,8,8a-octahydronaphthalen-1-one (solidagenone; **2**): This compound was obtained as a colorless crystal, with a melting point of 132–134 °C (water): $C_{20}H_{28}O_3$; HRMS-ESI: $[M+H]^+$ for $C_{20}H_{29}O_3$: 317.2111; found: 317.2106 *m/z* ESI-MS: 317.2111 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.00 (3H, s, 20-H), 1.15 (3H, s, 19-H), 1.19 (3H, s, 18-H), 1.36–1.16 (2H, m; 3-H), 1.59–1.53 (2H, m; 2-H), 1.78–1.58 (2H, m; 1-H), 2.02

(3H, d; J = 1.5 Hz, 17-H), 2.05–1.90 (2H, m; 11-H), 2.67–2.65 (2H, m; 12-H), 2.71 (1H, s; 5-H), 5.71 (1H, q; J = 1.5 Hz; 7-H), 6.30 (1H, dd; J = 1.8; 0.9 Hz; 14-H), 7.26 (1H, dd; J = 1.5; 0.9 Hz; 16-H), 7.37 (1H, dd; J = 1.8; 1.5 Hz; 15-H); ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 17.9 (C-2), 18.4 (C-20), 20.1 (C-17), 21.4 (C-12), 21.7 (C-18), 31.7 (C-1), 32.3 (C-4), 33.4 (C-11), 33.8 (C-19), 42.7 (C-3), 46.5 (C-10), 55.8 (C-5), 76.7 (C-9), 110.8 (C-14), 125.1 (C-13), 129.3 (C-7), 138.7 (C-16), 143.8 (C-15), 155.7 (C-8), 200.2 (C-6).



2

Antiproliferative effects of the extracts and the isolated compounds of Solidago chilensis

Both DES and HES inhibited cell proliferation with slightly different profile (Fig. 2). DES was more effective than HES in total growth inhibition, been U-251 cells the most sensitive (TGI = 33.24 μg/ml) (Table 1).

Besides, quercitrin (**1**), isolated from HES, was inactive (TGI > 250 μg/ml) while solidagenone (**2**), isolated from DES, showed a promisor antiproliferative activity inhibiting totally cell proliferation of U-251, MCF-7, 786-0, NCI-H460, and PC-3 cell lines at concentrations of 5.38 to 12.53 μg/ml (Fig. 3, Table 1).

Molecular properties of solidagenone

The molecular properties study was performed on basis of "Lipinski's rule of five" using the Molinspiration server. This same bioinformatics tool was used to obtain the bioactivity score. The results it is evident that solidagenone show quality binding energy values and also follow the Lipinski's Rule of Five (number of violations = 0). The bioactivity scores evidenced the predisposition of solidagenone to interact with nuclear receptors and enzymatic inhibitors (0.54 and 0.31, respectively) (Table 2).

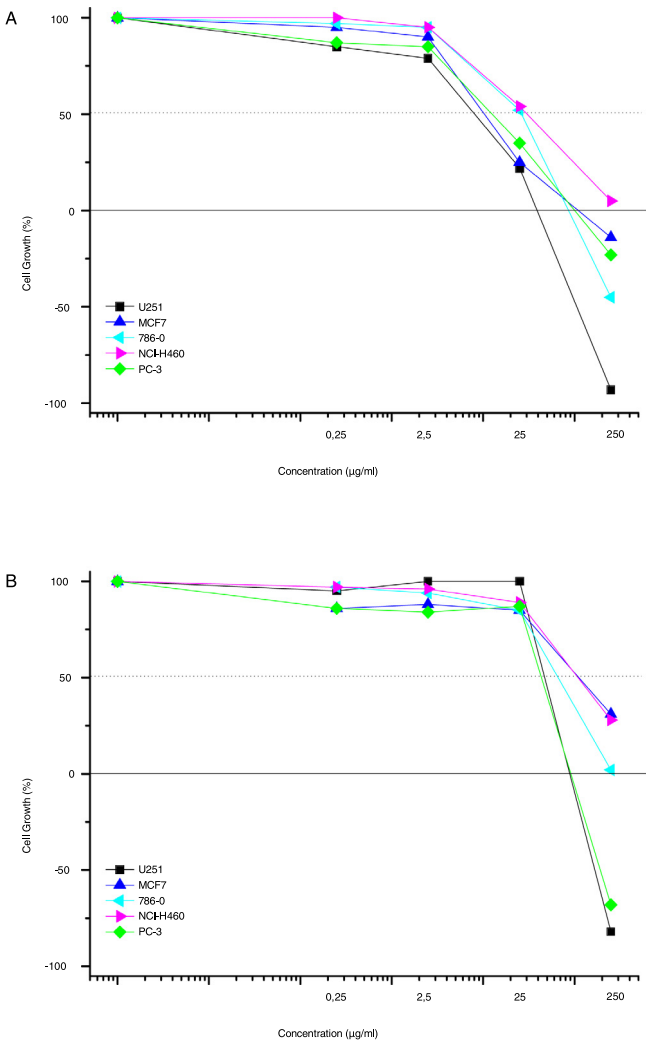


Fig. 2. Antiproliferative effects of dichloromethane (A) and hydroalcoholic (B) extracts of *Solidago chilensis* aerial parts. Concentration range: 0.25–250 μg/ml; exposition time: 48 h; human tumor cell lines: glioblastoma (U-251), breast (MCF-7), 786-O (kidney), non-small cells (NCI-H460), prostate (PC-3).

Discussion

Natural products obtained from animals, microorganisms and plants present great potential for the development of novel therapeutic agents (Hassig et al., 2014). These products are considered important sources of anticancer substances. It is estimated that 60% of all chemotherapeutic drugs are obtained, either directly or indirectly, from natural products (Newman and Cragg, 2016).

In this study, we prepared extracts from *S. chilensis* aerial parts with high and low polarity (HES and DES, respectively) identifying quercitrin (**1**) and solidagenone (**2**) from HES and DES, respectively. These results corroborated previous studies of *S. chilensis* roots, inflorescences and aerial parts (Torres et al., 1987; Schmeda-Hirshmann, 1988; Torres et al., 1989; Valverde-Soares et al., 2009; Roman Junior et al., 2015).

The flavonoids and diterpenes present in several medicinal species arouse considerable interest and may have chemopreventive and chemotherapeutic effects. Their mechanisms of action include deactivation of carcinogens, anti-proliferative effects, cellular division inhibition, induction of cell death and differentiation, angiogenesis inhibition, antioxidant effects, reversal of multidrug

Table 1
Antiproliferative activity (TGI, μg/ml) of hydroalcoholic and dichloromethane extracts and isolated compounds from *Solidago chilensis* aerial parts.

Cell lines	HES	DES	Quercitrin	Solidagenone	Doxorubicine
U-251	202.76	33.24	[a]	8.21	1.04
MCF-7	[a]	115.67	[a]	5.38	2.09
786-0	[a]	87.71	[a]	5.58	2.28
NCI-H460	[a]	[a]	[a]	12.53	0.89
PC-3	102.66	104.64	[a]	5.56	4.81

Human tumor cell lines: glioblastoma (U-251), breast (MCF-7), 786-O (kidney), non-small cells (NCI-H460), prostate (PC-3), TGI, total growth inhibition (μg/ml) after 48 h-exposition.

[a] Effective concentration higher than the highest tested concentration (250 μg/ml).

Table 2
Values of molecular properties and bioactivity score of solidagenone calculated through of server Molinspiration.

Property molecular	Bioactivity score	Solidagenone	
Molecular mass (u)	GPCR ligand	314.94	– 0.03
Partition coefficient (log *P*)	Ion channel modulator	4.22	0.04
Polar surface area (Å^2)	Kinase inhibitor	5.44	– 0.76
Number of hydrogen acceptors	Nuclear receptor ligand	3	0.54
Donor hydrogen atoms	Protease inhibitor	1	– 0.15
–	Enzime inhibitor	–	0.31

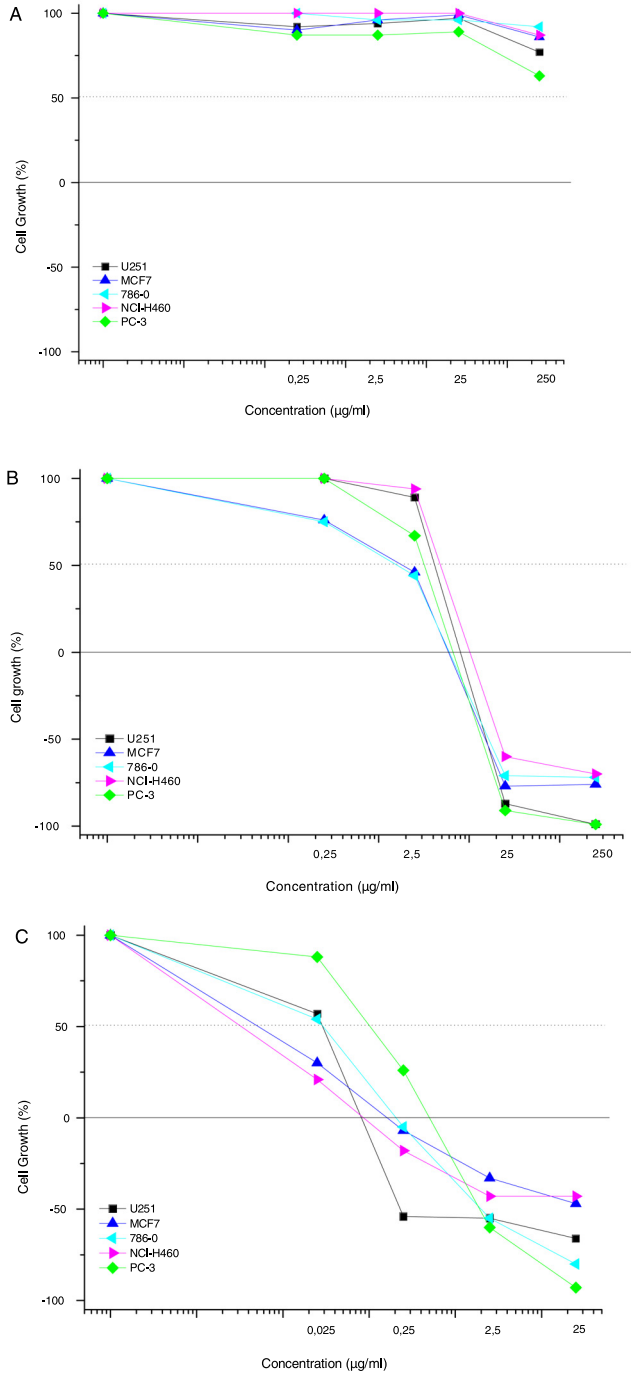


Fig. 3. Antiproliferative effects of quercitrin (A), solidagenone (B) and doxorubicine (C). Concentration range: 0.25–250 µg/ml; exposition time: 48 h; human tumor cell lines: glioblastoma (U-251), breast (MCF-7), 786-O (kidney), non-small cells (NCI-H460), prostate (PC-3).

resistance, or a combination of these mechanisms (Huang et al., 2012; Raffa et al., 2017).

According to Fouche et al. (2008), in the *in vitro* antiproliferative screening proposed by the National Cancer Institute (NCI) guidelines, extracts that showed TGI values equal or below to 50 µg/ml are promisor candidates. Using this criterium, in our study the HES was inactive while DES showed promisor antiproliferative effect against glioma cell line. This probably occurs due to increased lipophilic chemical constituents present in lower polarity extracts that results in higher affinity of these molecules to cellular membranes (Lee and Houghton, 2005).

There are a few descriptions of the antiproliferative effects of *Solidago* genus. The *S. microglossa* DC.leaves infusions was able induct to cytostatic effects, at 14 mg/ml, in the mitotic index method with *Allium cepa* (Bagatini et al., 2009). More, *S. virgaurea* L. rhizomes extract showed antitumor effect related to the presence of triterpenic saponins (Plohmann et al., 1997), while the diterpenes 6β-angeloyloxykolavenic acid and 6β-tigloyloxyolavenic acid, isolated from *S. canadensis* L. inflorescences, displayed cytotoxic activity against breast, cervix, leukemia, colon, and hepatoma tumor cell lines (Wu et al., 2008).

Unlike the results obtained by Cincin et al. (2014), who described the effects of 50 µM quercitrin against the NSCLC line (non-small cell lung cancer), we found that quercitrin did not inhibited any tumor cell line. In contrast, solidagenone isolated from the DES revealed very promissor TGI values specially against MCF-7 (breast), 786-0 (kidney) and PC-3 (prostate) cell lines (TGI < 6.25 µg/ml), that can be considered as potent antiproliferative activity according to Fouche et al. (2008), besides moderate cytostatic effect against U-251 and NCI-H460 cell lines (TGI = 8.21 and 12.53 µg/ml, respectively).

The "Rule of Five" proposed by Lipinski (2004) describes five theoretical parameters that molecules must possess in order to satisfy the pharmacokinetic aspects necessary to maximize the chance of success to become prototypes of new drugs. The molecular mass must be ≤500 atomic mass units (u), the partition coefficient (log *P*) must be ≤5, the polar surface area (PSA) should be ≤140 Å^2, number of hydrogen acceptors ≤ 10, and the molecule must also have a maximum of five donor hydrogen atoms. In this context, solidagenone has been found to satisfy all five theoretical parameters required to be a promisor drug candidate (314.94 u, log *P* = 4.22, 5.44 Å^2, 3 acceptors of hydrogen, one atom donor of hydrogen).

Moreover, using chemo-informatics tools (http://www.molinspiration.com) to evaluate probable affinity of a molecule for a given biological receptor (site of action), it was possible to observe the predisposition of solidagenone to interact with nuclear receptors and enzyme inhibitors (bioactivity score: 0.54 and 0.31, respectively). This interaction may in part help us understand the potent antitumoral activity of this compound and to propose further mechanistical studies.

Finnaly, the intraperitoneal LD_{50} of solidagenone has been described as higher than 600 mg/kg suggesting a "not harmful" molecule (Rodríguez et al., 2002).

The interest in natural products is increasing, especially those derived from plants, due to the increasingly high number of cancer cases worldwide (Rates, 2001; Jemal et al., 2009). In order to look for

new sources of therapeutic anticancer agents, many plant extracts and active principles have been studied in *in vitro* and *in vivo* cancer models, and the correlation of both studies became one of the key steps for the success of this type of research (Newman and Cragg, 2012; Marchetti et al., 2012). In this study, was observed strong effect antiproliferative *in vitro* to the solidagenone and the results make this molecule promising for antitumor activity assays *in vivo*.

Conclusions

The dichloromethane extract from the aerial parts of *S. chilensis* (DES) has a promisor antiproliferative effects *in vitro*. The major constituent of DES, the diterpene solidagenone showed a potent antiproliferative effect against human breast, kidney and prostate tumor cell lines. Solidagenone meets the theoretical physicochemical criteria for bioavailability of drugs and the observed biological effects were probably related to the interaction of the molecule with nuclear receptors and as an enzymatic inhibitor.

Authors' contributions

All authors contributed substantially to the work reported. DBG and WARJ conceived and designed the experiments, analyzed the data and wrote the paper; BZ, GL, RCB, CADV, APS, KAPD, GALZ, DM, JE, BO and PZ performed the experiments; MFCS, AB, TPB, ALTGR, PAGG, ASFM, VCF and JEC performed the experiments and contributed with materials, analysis tools and wrote the paper.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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