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Original article

Antiproliferative activity of spinasterol isolated of *Stegnosperma halimifolium* (Benth, 1844)

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ABSTRACT

Cancer is the major cause of death in the world, representing a significant public health problem. Plants have been shown as a great source of secondary metabolites with anticancer activity. The aim of this work was evaluated the antiproliferative activity of the methanolic extracts, chemical fractions and the compound spinasterol isolated of medicinal plant *Stegnosperma halimifolium*. The methanolic extracts of stem, leaf and stem/leaf was obtained by maceration. The methanolic extract of stem was purified by successive extractions with solvents as *n*-hexane, ethyl acetate and ethanol. The *n*-hexane fraction was separated by column chromatographic and monitored by thin layer chromatographic. The compound spinasterol was characterized by ^{1}H NMR, ^{13}C NMR and Mass Spectrometry. Methanolic extracts, chemical, chromatographic fractions and spinasterol was evaluated against RAW 264.7, M12.C3.F6, **PC-3**, LS-180, A549 and HeLa cancer cell lines by the standardized method MTT for determinate the antiproliferative activity. Methanolic extract of stem shown the better antiproliferative activity against the murine macrophage cancer cell line RAW 264.7. *n*-Hexane chemical fraction shown antiproliferative activity against human alveolar cancer cell line A549 and RAW 264.7. Was isolated and characterized a compound by NMR ^{1}H and ^{13}C, revealing the presence of sterol spinasterol. Spinasterol shown **to** have antiproliferative activity against cervical cancer cell line HeLa and RAW 264.7, **indicating** that spinasterol can be a responsible compound of antiproliferative activity found in the methanolic extract of *Stegnosperma halimifolium*.

1. Introduction

Currently a great number of infectious, pathologic and degenerative processes affect human health. Cancer in one of the major causes of dead worldwide, associated with 8.2 million of death in the world in 2012 (WHO, 2016); in Mexico is the second cause of dead in the country (INEGI, 2015). Approximately 60% of the world's population relies almost entirely on plants for medication and natural products have long been recognized as an important source of therapeutically effective medicines (Harvey, 2000). Mexico has approximately 4000 species of medicinal plants, however, the chemical, pharmacological and biomedical validation of the active principles has been carried out in only 5% of the species (Ocegueda et al., 2005). The ethnic population of the state of Sonora, located in the northwest of Mexico, has used approximately 450 different plants with medicinal purpose, **that** include endemic and **introduced** species, but only a fraction has the scientific validation of their medicinal properties.

The plants of the Stegnospermaceae family and the *Stegnosperma* genus include species unknown and poorly studied regarding their biological activities. Stegnospermaceae is a monogenic family that belongs to Cariophylalles order, the *Stegnosperma* genus is composed by 3 species, *watsoni*, *cubense* and *halimifolium*; it is widely distributed in the north of Mexico to Nicaragua and the

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Antilles (Johnson et al., 1996; Yetman and Van Devender, 2002). The plants of *Stegnosperma* genus are rich in betalains (Kubitzki et al., 1993), **also** the presence of glucosinolates (Vaughn and Berhow, 2005) and flavonoids like quercetin and kaempferol (Richardson, 1981) has been reported. *Stegnosperma halimifolium* (Benth, 1844) commonly called chapacolor or snake herb, is a shrub, with **oval** leaf, small white flowers (November to March season), and fruits like brown berries with black seed inside; it is found near to coast and hills. It is distributed mainly in Mexico, in the states of Sonora, Baja California, Sinaloa, Michoacan and Guerrero (Kubitzki et al., 1993; Yetman and Van Devender, 2002). The Mayo and Seri ethnic groups of Sonora State used this plant's leaf to heal snake bites or cure headache, tribes of Baja California **employed** the powder of roots like soap substitute.

Currently, little information is available regarding the biological activity of *Stegnosperma halimifolium*. Studies realized by Ortiz Sandoval (2010) show the antiproliferative potential of the methanolic extract of *Stegnosperma halimifolium*, against the HeLa, RAW 264.7, M12.C3.F6 and L-929 cell lines, with IC_{50} values of 200 μg/mL. Due to this, it is important to research the biological potential of the species of *Stegnosperma*, **therefore**, the aim of the present work was the chemical characterization of the compounds responsible for this activity in the methanolic extract of *Stegnosperma halimifolium*.

2. Material and methods

2.1. Plant material

The stem and leaves of *Stegnosperma halimifolium* were collected in La Manga, outside Hermosillo city, Sonora, Mexico (29°0 4′59.3″N, 111°03′02.9″W), in October 2014. Botanical specimens were identified by Professor Jesus Sanchez Escalante, head of Herbarium at the University of Sonora with voucher specimen no. 22658.

2.2. Preparation of methanolic extracts and isolation of spinasterol

The stem and leaves were dried in the shade at room temperature and powdered. The methanol extract was obtained by maceration of dried powder of *Stegnosperma halimifolium* in methanol for 10 days (Jiménez-Estrada et al., 2013). The solvent was removed by evaporation under reduced pressure at 60 °C in a rotary evaporator Butchi 850-V (Flawil, Switzerland). The methanolic extract of stem (that has the greatest antiproliferative activity) was consecutively partitioned with the following solvents: *n*-hexane, ethyl acetate and ethanol. The preparations obtained of fractionation process were concentrated on a rotary evaporator under reduced pressure at 60 °C. For the isolation of spinasterol, the *n*-hexane fraction was separated by column chromatography with silica gel as stationary phase and *n*-hexane, ethyl acetate and methanol like gradient; the chromatographic fractions were monitored by thin layer chromatography (TLC).

2.3. Cell lines cultures

L-929 (murine subcutaneous connective tissue), HeLa (human cervical carcinoma), A549 (human alveolar carcinoma), LS-180 (colorectal adenocarcinoma) and **PC-3** (prostate adenocarcinoma) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). RAW 264.7 (murine macrophage transformed by virus Abelson leukemia) and M12.C3.F6 (murine cell B lymphoma) were provided by Dr. Emil A. Unanue (Department of Pathology and Immunology, Washington University in St. Louis, MO). Cells were cultured in Dulbecco Medium Eagle Modified (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37 °C under a 5% CO_2 atmosphere.

2.4. Cell viability MTT assay

Cell viability assay was evaluated by the MTT [3-(4,5-dimethyl tiazol-2-yl)-2,5-diphenyltetrazolium] reduction assay (Mosmann, 1983) with some modifications (Hernandez et al., 2007). Briefly, cells (1×10^4 per well, 50 μL) were placed in each well of a 96-well plated. After 24 h incubation at 37 °C in a 5% CO_2 atmosphere to allow attachment, aliquots (50 μL) of medium containing different concentrations of extracts, fractions (400.0–12.5 μg/mL), chromatographic fractions and spinasterol (100.0–12.5 μg/mL), previously dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in the culture medium, were added and incubated for 48 h. Preliminary experiments established that the use of DMSO concentrations ranging from 0.06 until 2% in the cells cultures caused no cell damage. In the last 4 h of incubation, cells were washed with phosphate buffer solution (PBS) and fresh culture medium and 10 μL of a MTT solution (5 mg/mL) were added to each well. The cell viability was assessed by the ability of metabolically active cells to reduce the tetrazolium salt to colored formazan. The formazan crystals formed were dissolved in acidic isopropyl alcohol, and the absorbance of the samples was measured with an ELISA plate reader (Multiskan EX, Thermo Scientific, Waltham, MA, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Proliferative cells were expressed in terms of percentage, where the optical density measured form vehicle-treated cells was considered 100% of proliferation. Aqueous solution of doxorubicin was used as positive control with concentration range of 4.0–0.125 μg/mL. Antiproliferative activity of extracts, fractions and spinasterol was reported as IC_{50} values (defined as the concentration of extract required to inhibit cell proliferation by 50%) using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Values of proliferation of a least three experiments, with six doses, in triplicated, were log transformed, normalized and nonlinear regression analysis was used to generate a dose-response curve to calculated IC_{50} values.

2.5. Qualitative phytochemical screening

In order to identify the classes of phytochemicals present in the fractions obtained from the methanolic extract of stem of the *S. halimifolium*, a qualitative phytochemical screening was conducted. We used the next specific test: saponins (Rosenthaler test, foam test), cumarines (KOH test), glycosides (Keller-Kiliani test), sterols and terpenes (Liebermann-Buchnard), alkaloids (Wagner, Meyer test), tannins (gelatin test), flavonoids (NaOH 10% test), quinones (chloroform test), and phenol compounds (ferric chloride test) (Harborne, 1973; Gupta et al., 2013; Banu and Cathrine, 2015; Rascon-Valenzuela et al., 2015; Prabhavathi et al., 2016).

2.6. Chemical characterization of spinasterol

^1H, ^{13}C and bidimensional Nuclear Magnetic Resonance (NMR) experiments were performed on a Varian INOVA spectrometer, operating at 500 MHz in NMR ^1H and 125 MHz in NMR ^{13}C experiments. The samples were dissolved in deuterated chloroform ($CDCl_3$). Chemical shifts were given in δ ppm, as internal reference, residual not deuterated dissolvent $CHCl_3$ in δ 7.26 and tetramethylsilane (TMS) in δ 0 were used. Mass spectrometry (MS) experiments were performed on an MStation JMS-700 spectrometer (Electronic Impact (EI) ionization mode in positive mode, range of analysis 10–500 *m*/*z*, temperature 3276.7 °C).

2.7. Statistical analysis

The biological results were expressed as mean ± standard deviation of 3 repetitions. The difference of means was evaluated using an one way analysis of variance (one way-ANOVA) following by Tukey test using IBM SPSS statistics 20 **software** (IBM Inc., Hong Kong).

3. Results

3.1. Evaluation of antiproliferative activity of methanolic extracts of S. Halimifolium

In order to evaluate the antiproliferative activity of methanolic extract of stem, leaves and a mixture of leaves/stem of *Stegnosperma halimifolium*, these were tested against different cancer and non-cancer cell line. The concentrations range from 12.5 to 400.0 μg/mL on the human A549, **PC-3**, LS-180 and HeLa cancer cell lines, murine RAW 264.7 and M12.C3.F6 cancer cell lines, and the non-cancer murine L-929 cell line. The stem/leaves methanolic extract exhibited activity against the murine RAW 264.7 (IC_{50} = 243.12 μg/mL) and M12.C3.F6 (IC_{50} = 213.67 μg/mL) cancer cell lines, but not against the A549, HeLa, **PC-3**, LS-180 cancer cell lines and the non-cancer L-929 cell line. The methanolic extract of stem showed antiproliferative activity against the RAW 264.7 cell line with IC_{50} values of 118.09 μg/mL, but had no effect on the rest of the cell lines. No antiproliferative activity from the methanolic extract of leaves was present against both, the cancer and non-cancer cell lines (Table 1).

3.2. Evaluation of the fractions obtained of methanolic extract of stem

The methanolic extract of stem was fractioned with solvents of ascendant polarity, and the *n*-hexane, ethyl acetate and ethanol fraction were obtained. These were then evaluated for antiproliferative activity under the same conditions as previously described. The hexane fraction showed activity against the RAW 264.7 and M12.C3.F6 murine cancer cell lines with IC_{50} values of 65.9 and 146.1 μg/mL, respectively, and against the non-cancer L-929 cell line (IC_{50} = 133.4 μg/mL). This fraction showed a low effect on the HeLa cancer cell line with IC_{50} values of 343.2 μg/mL, but resulted active against the A549 cell line with IC_{50} values of 68.6 μg/mL, **PC-3** and LS-180 didn't show to be affect with the fraction. The ethyl acetate fraction exhibited antiproliferative activity against the RAW 264.7 and M12.C3.F6 murine cancer cell lines with IC_{50} values of 50.8 and 78.4 μg/mL, respectively, and low effect against the L-929 cell line (IC_{50} = 215.8 μg/mL). On other hand, this fraction showed a low effect against the A549 (IC_{50} = 349.7 μg/mL) and HeLa (IC_{50} = 133.4 μg/mL) cancer cell lines, but not affected to **PC-3** and **LS-180** cell line. No antiproliferative activity was present in the ethanolic fraction against any cell line tested (Table 2). Our results suggest that the responsible compound(s) of antiproliferative activity of *Stegnosperma halimifolium* correspond to those present in the low polarity-region of the methanolic extract of stem.

3.3. Qualitative phytochemical screening

How shown Table 3, the qualitative phytochemical screening of fractions obtained from the methanolic extract of *Stegnosperma halimifolium*, revealed the presence of sterols and terpenes in the *n*-hexane fraction, flavonoids in the ethyl acetate fraction and phenolic compounds, flavonoids and saponins in the ethanolic fraction. The results revealed that the compounds responsible of the antiproliferative activity of *Stegnosperma halimifolium* are terpenes and/or flavonoid compounds.

3.4. Antiproliferative activity of chromatographic fractions obtained from hexane fraction

The *n*-hexane fraction was fractioned by column chromatography, were 16 fractions were obtained and evaluated under the same conditions that the last experiments. Fractions 7, 9, 10 and 11 showed IC_{50} values of 60.4, 63.4, 55.2 and 57.4 μg/mL, respectively, against the A549 cancer cell line, shown a significant antiproliferative effect compare with the origin fraction, the hexanic fraction (Table 4). The chromatographic fraction 8 was obtained as a white powder, extracted with *n*-hexane/ethyl acetate 8:2 phase like gradient and was further characterized.

3.5. Chemical characterization of fraction 8

The chromatographic fraction 8 was obtained as a white, amorphous solid, with a melting point of 169 °C, soluble in chloroform and dichloromethane; in the TLC a defined purple band was revealed with phosphoric vanillin and didn't showed fluorescence at 254 or 365 nm.

In order to characterize fraction 8, NMR and MS experiments were carried out. The ^{1}H NMR revealed the presence of 3 signals with chemical displacement of 5.1, 5.09 and 4.98 ppm, indicating the presence of double bonds in the structure, 3.71 ppm signal revealed the presence of a proton joined at hydroxyl group. The ^{13}C NMR showed 4 signals at chemical displacements of 139.56, 138.15, 129.45 and 117.6 ppm indicating the presence of carbon: carbon double bonds, the 71.1 ppm signal represents a carbon atom connected with hydroxyl group.

Comparing the results of the ^{1}H and ^{13}C NMR with the literature, the compound with similar signals (Table 5) was identified as the sterol spinasterol (Fig. 1). HSQC experiments showed that chemical displacement signals 71.1 and 3.52 ppm were joined, corresponding to C3, 138.15 and 5.1 ppm represent the signals of C22, 129.45 and 4.98 ppm, 117.46 and 5.09 ppm represent signals of double bonds (C23 and C7 respectively), the 139.56 ppm signal wasn't joined to any proton signal, indicating the presence of qua-

Table 1
Evaluation of antiproliferative activity of methanolic extracts from *S. halimifolium* against human cancer cell line A549, HeLa, LS-180, PC-3; murine cancer cell line RAW 264.7, M12.C3.F6 and murine non-cancer cell line L-929.

Methanolic extract	IC_{50}[a] (μg/mL)						
	L-929	RAW 264.7	M12.C3.F6	HeLa	A549	LS-180	PC-3
Stem + Leaves	>400	243.12 ± 1.5*	213.67 ± 1.3*	>400	>400	>400	>400
Stem	>400	118.09 ± 1.75*	>400	>400	>400	>400	>400
Leaves	>400	>400	>400	>400	>400	>400	>400
Doxorubicin[b]	2.89 ± 0.11	0.38 ± 0.01	1.89 ± 0.06	>4	2.5 ± 1.4	3.8 ± 0.1	3.1 ± 0.3

[a] Half maximal inhibitory. The results are expressed as average ± Standard Deviation.
[b] Positive control.
* Significative differences ($p < 0.05$) comparing to L-929 cell line. Maximum concentration evaluated 400 μg/mL.

Table 2
Evaluation of antiproliferative activity of fractions generated from the methanolic extract of the stem of *S. halimifolium* against human cancer cell line A549, HeLa, PC-3, LS-180, murine cancer cell line RAW 264.7 and M12.C3.F6 and non-cancer cell line L-929.

Fraction	IC_{50}[a] (μg/mL)						
	L-929	RAW 264.7	M12.C3.F6	HeLa	A549	LS-180	PC-3
n-Hexane	133.4 ± 1.4	65.9 ± 1.1*	146.1 ± 1.4	343.2 ± 2.4	68.6 ± 1.3*	>400	>400
Ethyl Acetate	215.8 ± 2.1	50.8 ± 1.1*	78.4 ± 1.3*	130.9 ± 1.1	349.7 ± 1.2	>400	>400
Ethanol	NP	NP	NP	NP	NP	NP	NP
Doxorubicin[b]	2.89 ± 0.11	0.38 ± 0.01	1.89 ± 0.06	>4	2.5 ± 1.4	3.8 ± 0.1	3.1 ± 0.3

[a] Half maximal inhibitory. The results are expressed as average ± Standard Deviation.
[b] Positive control. NP: Not Activity Present.
* Significative differences ($p < 0.05$) comparing to L-929 cell line. Maximum concentration evaluated 400 μg/mL.

Table 3
Qualitative phytochemical analysis of the *n*-hexane, ethyl acetate and ethanol fraction obtained of the methanolic extract of stem of *Stegnosperma halimifolium*.

Test	*n*-hexane fraction	Ethyl acetate fraction	Ethanol fraction
Saponins			
Rosenthaler	–	–	–
Foam	–	–	+
Cumarines			
KOH	–	–	–
Alkaloids			
Wagner	–	–	–
Meyer	–	–	–
Tannins			
Gelatin	–	–	–
Flavonoids			
NaOH 10%	–	+	+
Quinones			
$CHCl_3$	–	–	–
Phenol compounds			
$FeCl_2$	–	–	+
Glycosides			
Keller – Kiliani	–	–	–
Terpenes			
Liebermann – Buchnard	+	–	–

(–) Negative test. (+) Positive test. Cholesterol for terpenes test, quercetin for flavonoids test, gallic acid for tannins and phenolic compound test and berberine for alkaloids test were used as positive control.

Table 4
Evaluation of antiproliferative activity of column chromatographic fractions of the *n*-hexane fraction of *S. halimifolium* against A549 human cancer cell line.

Chromatographic fraction	IC_{50}[a] (μg/mL)
1	>100
2	71.9 ± 1.08
3	>100
4	84.71 ± 1.32
5	81.13 ± 1.21
6	71.86 ± 1.29
7	60.45 ± 1.2
8	>100
9	63.4 ± 1.14
10	55.22 ± 1.09
11	57.39 ± 1.13
12	73.91 ± 1.17
13	84.65 ± 1.12
14	>100
15	96.48 ± 1.6
16	95.69 ± 1.19

[a] Half maximal inhibitory. The results are expressed as average ± Standard Deviation. Maximum concentration evaluated 100 μg/mL.

Table 5
Chemical displacements of ^{13}C and ^{1}H NMR of chromatographic fraction 8 and spinasterol.

Position	RMN ^{13}C (δ) ppm	RMN ^{1}H (δ) ppm	Spinasterol ^{13}C (^{1}H)
1	37.155	1.01, 1.74	37.1 (1.09, 1.82)
2	31.479	1.36, 1.72	31.5 (1.39, 1.77)
3	71.079	3.52 (1H, m)	71.1 (3.6, 1H, m)
4	37.997	1.18, 1.63	38.0 (1.27, 1.70)
5	40.275	1.33	40.3 (1.4)
6	29.69	1.15, 1.68	1.22, 1.74
7	117.46	5.09 (1H, m)	117.5 (5.18, 1H, m)
8	139.56	–	139.6
9	49.466	0.66	49.5 (1.66)
10	34.23	–	34.24
11	21.555	1.5	21.6 (1.48)
12	39.476	1.18, 1.92	39.6 (1.23, 2.0)
13	43.29	–	43.3
14	55.135	1.74	51.15 (1.81)
15	23.086	1.31, 1.44	23.0 (1.40, 1.52)
16	28.498	1.2, 1.66	28.5 (1.25)
17	55.918	1.18	55.95 (1.25)
18	12.049	0.5	12.05 (0.55)
19	13.037	0.72	13.0 (0.8)
20	40.817	1.97	40.8 (2.05)
21	21.372	1.5	21.4 (1.02)
22	138.158	5.1 (1H, dd)	138.15 (5.17, 1H, dd)
23	129.457	4.983 (1H, dd)	129.48 (5.09. 1H, dd)
24	51.253	1.45	51.26 (1.55)
25	31.874	1.48	31.9 (1.55)
26	21.079	0.75	21.2 (0.85)
27	18.99	0.72, 0.85	19.0 (0.84)
28	25.393	1.09, 1.33	25.4 (1.18, 1.42)
29	12.24	0.75	12.2 (0.81)

Chemical displacements expressed in (δ) ppm. Varian INOVA. 500 MHz (^{1}H), 125 MHz (^{13}C). $CDCl_3$. (dd): Double of double. (m): Multiplete.

ternary carbon. COSY experiments showed the correlation of proton signal at 5.1 and 4.98 ppm; confirms the presence of double bond with carbon atoms with displacement signals 138.15 and 129.45 ppm, corresponding to C22 and C23. The 1.18 and 1.97 ppm signals demonstrate the direct correlation of C17 and C20, carbons bound between the steran nucleus and the aliphatic lateral chain; also the correlation between the 3.52 and 1.63 ppm, corresponds to the bond of C3 and C4 (Fig. 2). The HMBC experiment showed the correlation of the 13.037 and 1.15 ppm corresponding to C19 and C6, joined to 3 bonds of distance, 12.049 and 1.18, 1.2 according to C18 to relation of C12 at two bonds distance and C16 at three bonds of distance. Signals at 21.372 and 5.1 ppm correlate the C21 joined to C22 at 2 bonds distance, 51.25 and 0.75 ppm corresponding to join between C24 and C29 at two bonds of distance, 31.87 and 0.85, 0.75 ppm represent the C25 with C27 at two bonds of distance and C29 at three bonds of distance. The 25.39 and 4.98-ppm signals represent the correlation of C28 and C23 at three bonds of distance, 12.24 and 1.09 ppm correspond to C29 joined to C28 at two bonds of distance (Fig. 3). DEPT 135 corroborated the presence of three cuaternarian carbons

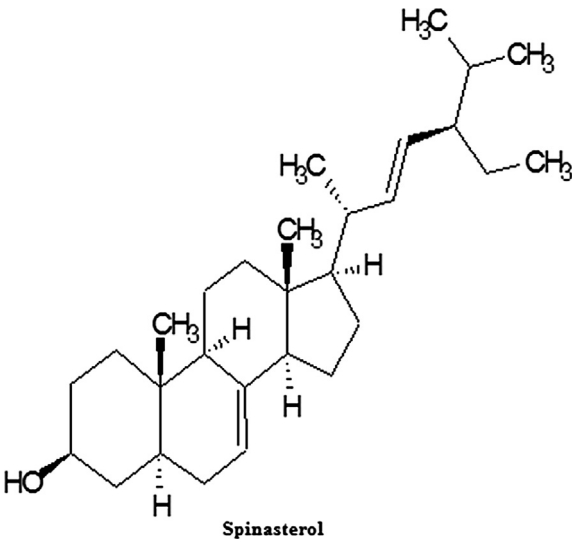

Fig. 1. Chemical structure of spinasterol.

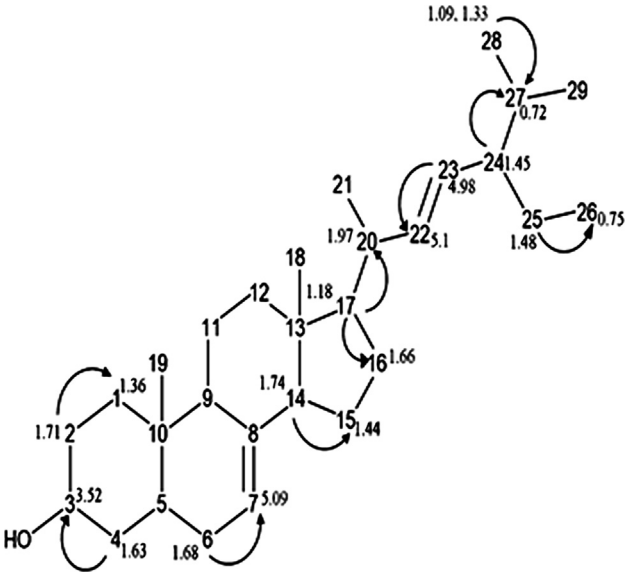

Fig. 2. COSY analysis for the identification of spinasterol.

to chemical displacement signals at 139.56, 43.292 and 34.232 ppm, corresponding to C8, C10 and C13 respectively, where C8 is bound to C7 via a double bond. The presence of nine -CH_2 groups at signals 39.47, 37.99, 37.15, 31.48, 29.65, 28.5, 25.4, 23.02 and 21.55 ppm correspond to C12, C4, C1, C2, C6, C16, C28, C15 and C11 respectively, coinciding with the spinasterol structure, 8 of the steran nucleus and 1 of the aliphatic chain.

MS spectrum revealed the molecular weight of the compound at 412 atomic mass units (AMU), with a fragmentation pattern of 81, 246, 255, 300 and 412, where, the loss of 141 AMU between the fragmentation peaks of 271–412 AMU correspond to the loss of the lateral aliphatic chain of sterol, and the loss of 271 AMU corresponding to steran nucleus.

3.6. Evaluation of antiproliferative activity of spinasterol

The isolated compound spinasterol was evaluated for antiproliferative activity against RAW 264.7, HeLa, A549, LS-180 and PC3 cancer cell lines, under the same conditions, with concentrations of 12.5–100.0 μg/mL for this experiment. No antiproliferative effect was present against the LS-180, A549 and PC-3 cell lines at the maximum concentration evaluated, however, spinasterol presented an antiproliferative effect against the HeLa cell line, with IC_{50} values of 77.1 μg/mL and the RAW 264.7 cell line, with IC_{50} values of 69.2 μg/mL (Table 6).

4. Discussion

The plants of genus *Stegnosperma* are poorly studied species regarding their phytochemical components and biological activities. Recent studies about antiproliferative activity of the methanolic extract of *Stegnosperma halimifolium* demonstrated the potential of this plant against cancer (HeLa, RAW 264.7 and M12.C3.F6 cell lines) with IC_{50} values between 200.0 and 400.0 μg/mL; no effect on the non-cancer cell line L-929 were reported. Similar to our results of the methanolic extract of stem/leaves against RAW 264.7 and M12.C3.F6 cell lines with IC_{50} values of 243.12 and 213.67 μg/mL respectively; the effect on the L-929 cell line was also similar as previously reported (Jiménez-Estrada et al., 2013). However, the IC_{50} values of methanolic extract of stem showed better activity against the RAW 264.7 cell line (118.09 μg/mL), so that this methanolic extract was further partitioned. It has been demonstrated that the purification process of methanolic extracts can enhance the **biological** activity in relation to the original extract; the techniques mostly used is with the extraction with solvents with different polarities like *n*-hexane, dichloromethane, ethanol or water, High Performance Liquid Chromatography (HPLC), column chromatography or supercritical fluid. (Cannell, 1998; Colegate and Molyneux, 2008).

In the present work, was used the solvent partition, obtaining three fractions: *n*-hexane, ethyl acetate and ethanol. The antiproliferative activity of *n*-hexane and ethyl acetate fractions was enhanced in relation with the initial methanolic extract of stem, with activity on cell lines that not present on the methanolic extract. For instance, against HeLa and A549 cell lines, with IC_{50} values of 343.2 and 68.6 μg/mL respectively, for *n*-hexane fraction, IC_{50} values of 130.9 and 349.7 μg/mL respectively, for ethyl acetate fraction. The effect on the RAW 264.7 cell line was also enhanced to IC_{50} values of 65.9 and 50.8 μg/mL respectively, for *n*-hexane and ethyl acetate fractions.

The chromatography fraction 8 was obtained by column chromatography of the *n*-hexane fraction, where the compound spinasterol was identified (Choi et al., 2003; Ragasa and Lim, 2005; Zhang et al., 2005; Muthia and Andalas, 2015). The phytochemical screening showed a positive result for terpenes and sterols, previous studies on *Stegnosperma halimifolium* have reported the presence of sterol compounds like β-sitosterol and spinasterol (Xu and Patterson, 1990). Spinasterol was isolated for first time from the spinach, and numerous references exist about the isolation of spinasterol in medicinal plants in families near to Stegnospermaceae, like Polygalaceae, Cucurbitaceae, Phytolaccaceae, among others. Spinasterol has demonstrated different biological activities including antiulcerogenic (Klein et al., 2010), antidepressant (Socała et al., 2015; Socała and Wlaź, 2016), anti-inflammatory (Jeong et al., 2010; Borges et al., 2014), antigenotoxic, antitumor and antiproliferative (Villaseñor et al., 1996; Ntie-kang and Yong, 2014).

Several studies exist about of antiproliferative activity of spinasterol against various cancer cell lines, **where** spinasterol has showed a particular behavior. The results in the present work indicate that spinasterol didn't shown antiproliferative activity against the cancer cell lines A549, LS-180 and **PC-3** to the experimental concentrations. Similar results was obtained in other researches, Cimmino et al. (2016) isolated spinasterol of the plant

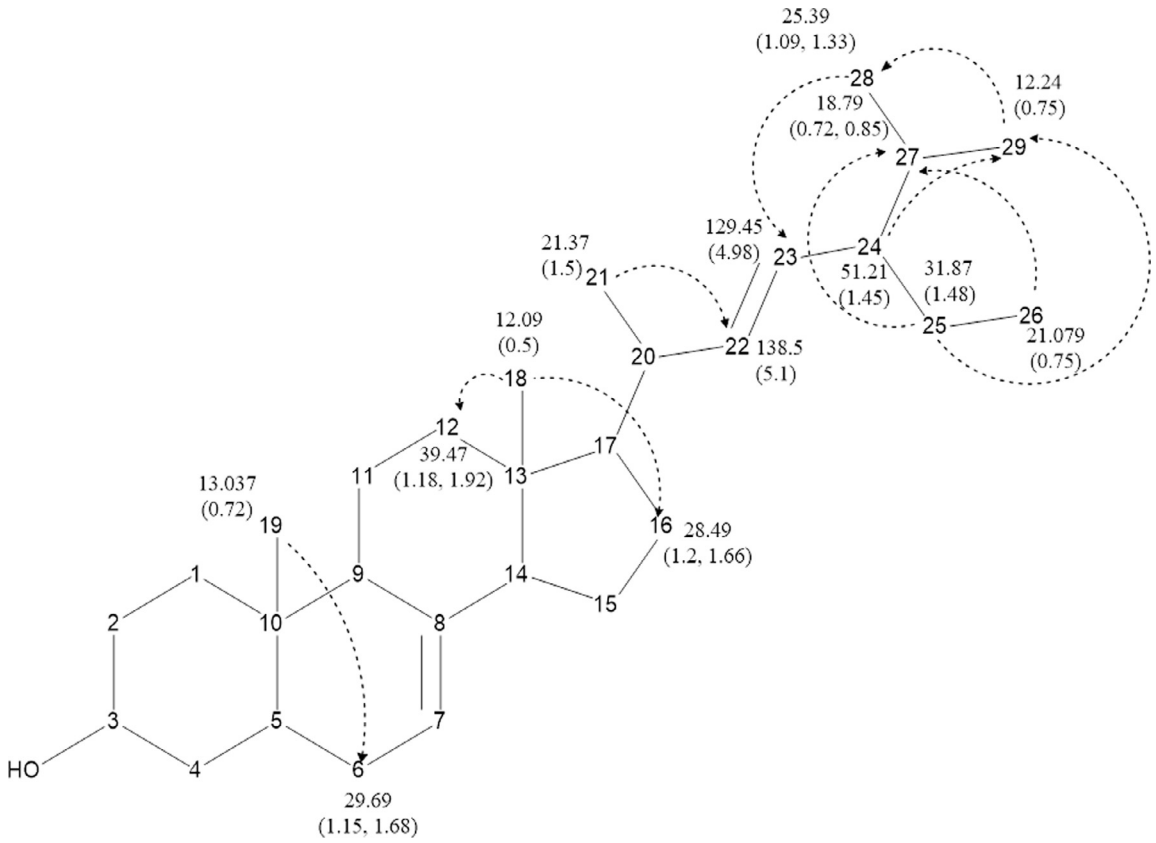

Fig. 3. HMBC analysis for the identification of spinasterol.

Table 6
Evaluation of antiproliferative activity of spinasterol against human cancer cell lines LS-180, A549, PC-3, HeLa and murine cancer cell line RAW 264.7.

Cell line	IC_{50}[a] (μg/mL) Spinasterol
LS-180	>100
A549	>100
PC-3	>100
HeLa	77.1 ± 2.1
RAW 264.7	69.2 ± 6

[a] Half maximal inhibitory. The results are expressed as average ± Standard Deviation. Maximum concentration evaluated of 100 μg/mL.

Impatients glandulifera where, this compound didn't show an effect in the cell lines A549, SKMEL-28 and U373 at the maximum concentration evaluated. Spinasterol isolated of green asparagus (*Asparagus officinalis* L.) didn't have inhibitory effect in the murine hepatic stellate cells t-HSC/CI-6 cell line exhibit IC_{50} values up to 200 μM (Zhong et al., 2015). In other hand, our results showed an antiproliferative effect of spinasterol against cervical cancer cell line HeLa and murine macrophage cell line RAW 264.7. Diverse authors reporting that spinasterol has potential as an antiproliferative and antitumor compound (Villaseñor and Domingo, 2000); different studies in cell lines corroborated this effect. Isolation of spinasterol from the methanolic extract of *Polyalthia cerasoides* seeds was evaluated against the CACO-2 cell line (colorectal cancer), where spinasterol showed antiproliferative activity (Ravikumar et al., 2010). Studies of spinasterol obtained of argan oil and cactus pear seed oil, expose the capacity of this compound to inhibit the cellular grown in the microglial murine cell line BV2, with IC_{50} values between 80 and 155 μM (Kharrasi et al., 2014). Spinasterol isolated from *Conyza canadensis* showed antiproliferative activity against the HeLa, MCF-7, A431 and MRC-5 cancer cell lines (Csupor-Loaffer et al., 2011). Studies on the effect of the methanolic extract of *Pueraria mirifica* roots, where spinasterol was the final identified compound **and evaluated** against cell lines of gynecological origin, **exhibited** a strong antiproliferative effect against breast cancer (MCF-7 and MDA-MB-231), ovarian (2774) and cervical (HeLa) cell lines was observed. Probably through a phytoestrogen-like effect, as agonist of the estrogen E_2 receptor (estradiol) with affinity to estrogen receptor ERα, experimental conditions shown that cervical cancer cell line HeLa and breast cancer cell line MCF-7 overexpressing estrogen receptor ERα (Jeon et al., 2005). The signaling system of the estrogen receptors (ER) plays a crucial role on the reproductive characteristics in the organism. ERα and ERβ are the most common receptors, both found in different tissues of humans and mice: the central nervous, intestinal and immune systems, liver, bones, breast, cervix, prostate, colon, and kidneys (Rollernova and Urbancinkova, 2000; Vegeto et al., 2003). It has been demonstrated that the interaction of some phytoestrogens with ERβ helps to prevent colorectal cancer (Barone et al., 2008). Also, reports exists that in the breast tissue, while the ERβ levels are controlled in normal cells, the levels of these are reduced in tumor cells (Xiong et al., 2016). The phytoestrogen are chemical derivates of plants with estrogenic activity, some phytoestrogens act like agonist, stabilized the conformation of the receptors, facility the activation of transcription (Bai and Gust, 2009). Maybe spinasterol act like phytoestrogen, but is necessary more studies that evidence this mechanism.

5. Conclusions

The results indicate that the responsible compounds of the antiproliferative activity of methanolic extract of *S. halimifolium*

are present in the non-polar fractions. Spinasterol compound was isolated of the *n*-hexane fraction obtained of the methanolic extract of stem and proved to be a compound with antiproliferative effect in the cervical cancer cell line HeLa and murine macrophage cancer cell line RAW 264.7, being one of the responsible compounds of the **biological** activity of methanolic extract of stem of *S. halimifolium*.

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