

Cytotoxicity and chromatographic analysis of *Dioon spinulosum*, family Zamiaceae

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ABSTRACT

The identification of cytotoxic secondary metabolites from *Dioon spinulosum* Dyer ex. leaves was our aim. Thus, the evaluation of the cytotoxic activity of the total alcohol extract and successive fractions [*n*-hexane, dichloromethane (DCM), ethyl acetate, and *n*-butanol] against endocervix carcinoma (HeLa) and breast cancer (MCF7) cell lines was carried out using the sulforhodamine B assay. Identifying and authenticating *D. spinulosum* Dyer by DNA fingerprinting were carried out using Start Codon Translation (SCoT) and Inter simple Sequence Repeat, and revealed that SCoT4, SCoT6, SCoT8, HB-9, and HB-14 can be used for the identification of *D. spinulosum* at the genetic level. Chromatographic analysis and isolation, followed by the spectroscopic detection of the isolated compounds, were achieved by using various spectroscopic techniques. The following five compounds were isolated: β -sitosterol (1), 7,7'',4',4'''-*O*-tetra-methylamentoflavone (2), sciadopitysin (3), amentoflavone (4), and aromadendrin (5). Compounds 1 and 5 were obtained for the first time from the titled plant. This plant showed good cytotoxic activity against endocervix carcinoma as the total ethanolic extract, *n*-hexane, DCM, ethyl acetate, and *n*-butanol fractions showed IC₅₀ values of 21.8, 21.1, 24.5, 26.5, and 21.4 μ g/ml respectively, against HeLa cell lines. On the other hand, the total ethanolic extract and the fraction of ethyl acetate showed good anticancer activity against MCF7 cell line with IC₅₀ values of 22.5 and 18 μ g/ml, respectively. Aromadendrin showed IC₅₀ = 12.5 μ g/ml against MCF7 cell line. In conclusion, aromadendrin may be responsible for *D. spinulosum* cytotoxicity against breast cancer cell lines. The total extract exhibited a promising adjunctive therapy for anticancer patients with endocervix and breast carcinomas.

INTRODUCTION

Plant-derived compounds have an undeniable role in the treatment of various diseases, including cancer. Several approved antitumor drugs have been extracted from plants, and several other compounds are in cancer clinical trials (Tayarani-Najaran and Emami, 2011). Many studies have showed that plant species are being used for cancer treatment or prevention of cancer development, especially in developing countries (Fouché *et al.*, 2008; Ochwang'I *et al.*, 2014). Cycads are plants belonging to class Gymnospermae, order Cycadales. The seeds of cycads in

many parts of the world are used in the form of flour to produce many edible products (Nair and Van-Staden, 2012). *Dioon* genus is one of the cycads that belongs to the family Zamiaceae and is represented in Egypt by only two species: *D. spinulosum* Dyer ex. Eichler and *D. edule* Lindl (Hamdy *et al.*, 2007). *D. spinulosum* Dyer ex. Eichleris is native to Mexico and South Africa and superficially resembles a palm (Johnson and Wilson, 1990). Being a member of cycads, *Dioon* genus has biflavonoids as secondary metabolites (Harborne, 1977). Amentoflavone-type biflavonoids, such as amentoflavone, bilobetin, sequoiaflavone, isoginkgetin, sciadopitysin, 7,4',7'',4'''-tetra-*O*-methyl amentoflavone, and diooflavone (amentoflavone hexamethyl ether), were previously reported (Dossaji *et al.*, 1975). It also has C-glycosylflavones, which were detected by Thin Layer Chromatography (TLC) (Carson and Wallace, 1972). On the other hand, hinokiflavone and its derivatives is absent from the whole family (Meurer and Stevenson, 1994).

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Dioon spinulosum has many reported bioactivities, especially cytotoxic activities. It is traditionally used in Mexico for the treatment of ulcers (Mena-Rejon *et al.*, 2009), gastric cancer, and gastrointestinal disorders (Alonso-Castro *et al.*, 2011). The methanolic extract of *D. spinulosum* petioles showed good *in vitro* cytotoxic activity on Hep-2 cell line ($IC_{50} = 20 \mu\text{g/ml}$) among four tested cell lines: nasopharynx carcinoma (KB), laryngeal carcinoma (Hep-2), cervix adenocarcinoma (HeLa), and cervix squamous carcinoma cells (SiHa) (Mena-Rejon *et al.*, 2009). In search for a potential treatment for estrogen-dependent tumors, a screening of five cycads including *D. spinulosum* as inhibitors of the cytochrome P-450 aromatase was carried out, and the ethyl acetate-derived fraction from *D. spinulosum* Dyer showed 97% aromatase enzyme inhibition (Kowalska *et al.*, 1995). The total extract was used in the previous studies without chromatographic separation of the secondary metabolites in search for the bioactive principles in *D. spinulosum*.

In this study, the evaluation of the cytotoxic activity of total extract and fractions against different cancer cell lines, including endocervix carcinoma (HeLa) and breast cancer (MCF7) cell lines, was carried out, followed by chromatographic isolation in order to identify the chemical constituents of the active fractions using Gas Chromatography-Mass Spectrometry (GC-MS) analysis and column chromatography.

EXPERIMENTAL STUDY

General experimental procedures

Genetic profiling of *D. spinulosum* Dyer was conducted in the National Research Center, Dokki, Giza, using Inter simple Sequence Repeat (ISSR) and Start Codon Translation (SCoT) markers.

GC-MS analysis of unsaponifiable matter (USM) was carried out using Agilent's gas-liquid chromatography, series 6,890, fitted with a flame ionization detector (FID). ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) were carried out on 400 MHz and 100 MHz, respectively, and were recorded on Bruker Avance III 400 MHz (Bruker AG, Fällanden, Switzerland) with AEON Nitrogen-Free Magnet and BBFO Smart Probe. Data acquisition and processing were carried out using Topspin 3.1 software.

For chromatography, silica gel 60 for CC precoated TLC plates (silica gel: 60 F_{254}) (Merck Darmstadt, Germany), Sephadex LH-20 (Pharmacia Uppsala, Sweden), and 1% *P*-anisaldehyde's reagent were used. The chemicals for cytotoxicity assay were dimethyl sulfoxide, RPMI-1640 medium, trypan blue, fetal bovine serum, penicillin/streptomycin antibiotic, trypsin-EDTA (Sigma Aldrich), and Tris buffer (AppliChem). All chemicals and reagents used in this study were of analytical grade.

Plant material

Dioon spinulosum Dyer ex. Eichler leaves were collected in the years from 2015 to 2016, from the Orman Garden in Cairo, Egypt. The plant was authenticated by Dr. Reem Samir, Professor of Botany, Faculty of Sciences, Cairo University, Egypt. The voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University. The leaves were dried in an oven at a temperature not exceeding 40°C, pulverized, and kept for further study.

Extraction and fractionation

Dioon spinulosum leaves (1 kg) were powdered and extracted by cold maceration with 70% ethanol until exhaustion. The solvent was evaporated under reduced pressure at 45°C to yield 50 g residue. Around 45 g of the residue was suspended in 100 ml of distilled water and extracted successively with *n*-hexane, dichloromethane (DCM), and ethyl acetate, and then *n*-butanol was saturated with water. Different fractions were evaporated separately under decreased pressure to yield 6, 10, 6, and 5 g residues, respectively.

Genetic profiling

The bulked DNA extraction was carried out using DNeasy Plant Mini Kit (QIAGEN) as mentioned by Rachmayanti *et al.* (2006), followed by polymerase chain reaction for SCoT and ISSR analysis, and gel electrophoresis methods were carried out as mentioned by Collard and Mackill (2009).

Chromatographic isolation

Preparation of USM

Preparation of the USM for Gas Liquid Chromatography (GLC) analysis was conducted as that mentioned in the Egyptian Pharmacopoeia (2005). The hexane extract (6 g) of *D. spinulosum* Dyer ex. leaves was refluxed with 80 ml alcoholic potassium hydroxide (10%) for 24 h, then concentrated, diluted with water, and extracted with diethyl ether, until removal of the unsaponifiable matter to yield 5.5 g USM.

GC-MS analysis of USM

This analysis was carried out by using Agilent's gas-liquid chromatography, series 6,890, fitted with FID detector. Identification of the components was carried out by comparing their retention times, retention indices, and mass spectral fragmentation patterns with those of the available references and/or published data (Adams, 2007), and identification was carried out through MS libraries (NIST, Wiley and PubChem).

Chromatographic separation of USM

A weighed amount of unsaponifiable matter (3 g) was subjected to fractionation by silica gel column (150 g, 5 × 70 cm). Elution was initiated with 100% *n*-hexane and the polarity gradually increased with EtOAc in 10% stepwise increment to 100%. Fractions (25 ml each) were collected, concentrated, and monitored by TLC. Similar fractions were pooled together. The subfractions (18–30) eluted with 20%–30% EtOAc in *n*-hexane were pooled and dried to yield 700 mg fraction. It was further purified on Sephadex LH-20 column (2 × 75 cm) and isocratic elution DCM-MeOH (95:5) to yield compound 1 (200 mg).

Investigation of DCM fraction

DCM extract of *D. spinulosum* leaves (8 g) was chromatographed on a silica gel column (5 × 75 cm) using DCM-MeOH with a 5% increment, collecting 25 ml fractions that were monitored by TLC. Similar fractions were pooled together to give three main subfractions. Fraction A (0.25 g, eluted with 5% MeOH in DCM) was rechromatographed on a silica gel column (1 × 60 cm) eluted isocratically with DCM-MeOH

(95:5) to yield compound **2** (8 mg). Fraction B (0.5 g, 25–39, eluted with 10%–15% MeOH in DCM) was rechromatographed on a silica gel column (1 × 70 cm) eluted with 100% DCM; then the polarity increased with 1% MeOH to yield compound **3** (5 mg). Fraction C (2 g, eluted with 20% MeOH in DCM) was rechromatographed over Sephadex LH-20 (2 × 60 cm) eluted with DCM-MeOH (80:20) to yield compound **4** (300 mg).

Investigation of EtOAc fraction

The EtOAc extract of *D. spinulosum* Dyer ex. leaves (5 g) was chromatographed on a polyamide column (5 × 70 g) eluted with 100% H₂O and the polarity gradually decreased with MeOH in 20% stepwise increments to 100% MeOH. Fractions (25 ml each) were collected and monitored by TLC. The subfraction eluted with 20% MeOH was rechromatographed over Sephadex LH-20 column (2 × 75 cm), eluted with 20% H₂O in MeOH to yield compound **5** (10 mg).

In-vitro cytotoxic activity

D. spinulosum Dyer ex. leaves, total ethanolic extract, and its four fractions were evaluated for cytotoxicity using the sulforhodamine B (SRB) assay in MCF7 cell lines (human breast adenocarcinoma) and HeLa (human endocervix carcinoma). The cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Minnesota, MN) and were maintained by serial subculture at the National Cancer Institute in Cairo, Egypt. The study was carried out according to the SRB assessment (Vichai and Kirtikara, 2006). The relationship between the surviving fraction and the concentration of the drug is designed to reach the survival curve of each tumor cell line after the defined compound. The IC₅₀ (dosage of the sample which reduces survival to 50%) was calculated for each extract and cell line.

RESULTS

DNA analysis

Genetic diversity revealed by SCoT markers

Five SCoT polymorphic markers were screened to access the level of genetic diversity and similarity between two *Dioon* species which grow in Egypt. They yielded 31 scorable bands as shown in Table 1. The bands ranged from 275 to 2,360 bp and the band numbers varied from two to seven in each primer. The total polymorphism was estimated to be 45.16%. The distribution of monomorphic (common) and polymorphic bands generated by the five SCoT markers showed 14 unique markers, both positive and negative markers; two unique bands were obtained from *D. spinulosum* with primer SCoT2; one of them was positive and the other was negative with a molecular size of 370 and 630, respectively. Three positive unique bands were obtained from *D. spinulosum* with primer SCoT4 with a molecular size of 730, 930, and 1,500 bp. Four unique bands were obtained from *D. spinulosum* with primer SCoT6; two of them were positive with a molecular size of 600 and 760 bp, and the others were negative with a molecular size of 700 and 2,360, respectively. Five unique bands were obtained from *D. spinulosum* with primer SCoT8; two of them were positive with a molecular size of 275 and 530 and the others were negative with a molecular size of 300, 800, and 1,000 bp, respectively.

Genetic diversity revealed by ISSR markers

In the case of ISSR markers, five ISSR polymorphic markers were screened to access the level of genetic diversity and similarity between two *Dioon* species which grow in Egypt. They yielded 30 scorable bands. The bands ranged from 240 to 1,860 bp and the band numbers varied from three to eight in each primer. The total polymorphism was estimated to be 50% as shown in

Table 1. The distribution of monomorphic and polymorphic bands generated by the SCoT markers.

Marker name	Total bands	Monomorphic bands	Polymorphic bands	Unique bands	% polymorphic
SCoT2	5	3	2	2	40%
SCoT 3	3	3	–	–	–
SCoT 4	5	2	3	3	60%
SCoT 6	9	5	4	4	44.44%
SCoT 8	9	4	5	5	55.55%
Total	31	17	14	14	45.16%

Table 2. The distribution of monomorphic and polymorphic bands generated by the ISSR markers.

Marker name	Total band	Monomorphic band	Polymorphic band	Unique band	% polymorphic
14A	5	5	–	–	–
44B	5	4	1	1	25%
HB-9	6	2	4	4	66.66%
HB-12	5	3	2	2	40%
HB-14	9	6	3	3	33.33%
Total	30	20	10	10	33.33%

Table 2. The distribution of monomorphic and polymorphic bands produced by the five ISSR markers revealed that primer 14A did not produce any unique marker with *Dioon* species. Primer 44B showed one negative unique band with *D. spinulosum* with molecular size 860 bp. Primer HB-9 showed three positive unique bands with *D. spinulosum* with a molecular size of 780 and 560 bp and one negative band with a molecular size of 400 bp. Primer HB-12 produced two positive unique bands with *D. spinulosum* with a molecular size of 670 and 980, respectively. Primer HB-14 produced one positive unique band with a molecular size of 240 bp and two negative unique bands with a molecular size of 1,500 and 1,860 bp, respectively.

SCoT and ISSR markers gave a total of 61 scorable bands. The SCoT markers yielded 31 scorable bands and they ranged from 275 to 2,360 bp and the band numbers varied from 2 to 7 in each primer and showed 14 unique bands, and the total polymorphism was estimated to be 45.16%. The ISSR markers yielded 30 scorable bands, these bands ranged from 240 to 1,860 bp, and the band numbers varied from 3 to 8 in each primer; the total polymorphism was estimated to be 33.33%. The proportion of polymorphism indicates that the examined cultivars exhibit a moderate level of polymorphism.

Column chromatography

The phytochemical study of *D. spinulosum* plant resulted in the isolation of five compounds; the structures of the isolated compounds were elucidated using ¹H-NMR and DEPT-Q

experiments, in addition to the comparison with published data. The isolated compounds were identified as β -sitosterol (1) (Nyigo *et al.*, 2016), 7,7'',4',4'''-O-tetra-methyl amentoflavone (2) (Moawad and Amir, 2016), sciadopitysin (3) (Wollenweber *et al.*, 1998), amentoflavone (4) (Lee *et al.*, 2011), and aromadendrin (5) (Chunhakant and Chaicharoenpong, 2019). Compounds 1 and 5 were being reported for the first time in *D. spinulosum*; the structure of the isolated compounds is shown in Figure 1, and ¹H NMR data of isolated flavonoids were compiled in Table 3.

GLC analysis

The unsaponifiable matters of *D. spinulosum* Dyer ex. were dominated by a large amount of hydrocarbons, 18 compounds, and represent 75.02%, including 1-butylhexyl benzene 4.42%, 1-propylheptyl benzene 3.63%, 1-ethyloctyl benzene 2.49%, 1-methylnonyl benzene 2.54%, 1-propyloctyl benzene 7.25%, 1-ethylnonylbenzene 5.8%, 1-methyldecylbenzene 7.18, 1-butyloctyl benzene 4.54%, 1-propylnonyl benzene 6.02%, 1-ethyldecyl benzene 4.34%, 1-methylundecyl benzene 5.94%, 1-pentyloctyl benzene 5.98%, 1-butylnonyl benzene 4.17%, 1-propyldecyl benzene 4.13%, 1-ethylundecyl benzene 2.30%, 1-methyldodecyl benzene 3.51%, heneicosane 0.2%, and pentatriacontane 0.6%. Sterols, 6 compounds, represent 12.21%, including β -sitosterol 3.32%, stigmastan-3,5-diene 2.44%, pregnan-20-one,3-(acetyloxy)-5,6:16,17diepoxy 2.79%, androstane-11,17-dione,3-[(trimethylsilyl)oxy]-17-[o-(phenylmethyl)oxime] 3.62%, stigmastan-6,22-dien,3,5-diedihydro 0.02%, and 4,6-cholestadienol 0.02%. Terpenes were

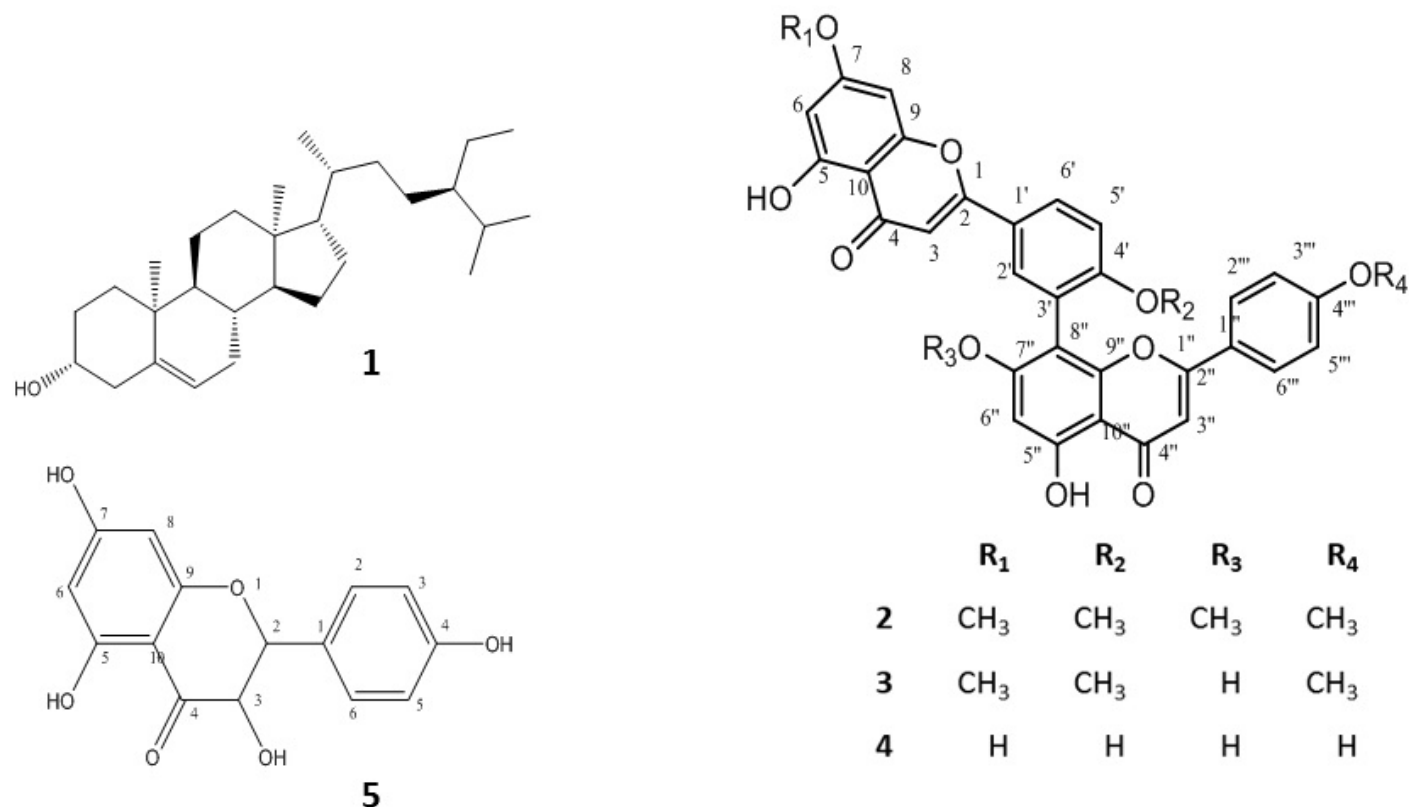


Figure 1. Compounds isolated from *D. spinulosum* leaves.

Table 3. ¹H NMR data of isolated flavonoids (compounds 2–5) at 400 MHz.

H	δ_H , mult, J value			
	2	3	4	5
2	—	—	—	5.00 (d, J = 11.4 Hz)
3	6.80 (s)	6.8 (s)	6.66 (s)	4.56 (d, J = 11.6 Hz)
6	6.32 (d, J = 2.1 Hz)	6.28 (d, J = 2.1 Hz)	6.24 (d, J = 2.1 Hz)	5.90 (d, J = 1.8 Hz)
8	6.61 (s)	6.65 (d, J = 2.1 Hz)	6.53 (d, J = 2.1 Hz)	5.94 (d, J = 1.8 Hz)
3''	6.73 (s)	6.68 (s)	6.72 (s)	—
6''	6.66 (s)	6.44 (s)	6.47 (s)	—
2'	8.13 (d, J = 2.2 Hz)	8.11 (d, J = 2.3 Hz)	8.1 (d, J = 2.2 Hz)	7.37 (d, J = 8.4 Hz)
3'	—	—	—	6.8 (d, J = 8.4 Hz)
5'	7.39 (d, J = 8.6 Hz)	7.36 (d, J = 8.8 Hz)	7.26 (d, J = 8.5 Hz)	6.8 (d, J = 8.4 Hz)
6'	8.20 (dd, J = 8.6, 2.3 Hz)	8.17 (dd, J = 8.8, 2.3 Hz)	8.01 (dd, J = 8.8, 2.3 Hz)	7.37 (d, J = 8.4 Hz)
H-2'' & H-6''	7.67 (d, J = 8.2 Hz)	7.65 (d, J = 8.6 Hz)	7.63 (d, J = 8.5 Hz)	—
H-3''' & H-5'''	6.94 (d, J = 8.5 Hz)	6.91 (d, J = 8.6 Hz)	6.83 (d, J = 8.5 Hz)	—
5-OH	12.95 (s)	12.90 (s)	12.97 (s)	—
5''-OH	13.26 (s)	13.08 (s)	13.14 (s)	—
4'-OCH ₃	3.86 (s)	3.83 (s)	—	—
4'''-OCH ₃	3.91 (s)	3.77 (s)	—	—
7-OCH ₃	3.88(s)	3.85 (s)	—	—
7''-OCH ₃	3.80 (s)	—	—	—

NMR spectra were run in acetone-*d*₆, except for compound **5** which was run in MeOD.

Table 4. Identified compounds in GC/MS analysis of the unsaponifiable matter of *D. spinulosum* leaves.

Peak number	Compound name	RRt*	Area%	Molecular weight
1	1-Butylhexyl benzene	0.87	4.42	218.3
2	1-Propylheptyl benzene	0.88	3.63	218.3
3	1-Ethylloctyl benzene	0.91	2.49	218.3
4	1-Methylnonyl benzene	0.95	2.54	218.3
5	1-Propyloctyl benzene	1	7.25	232.4
6	1-Ethylnonyl benzene	1.02	5.8	232.4
7	1-Methyldecyl benzene	1.06	7.18	232.4
8	1-Butyloctyl benzene	1.1	4.54	246.4
9	1-Propylnonyl benzene	1.11	6.02	246.4
10	1-Ethyldecyl benzene	1.13	4.34	246.4
11	1-Methylundecyl benzene	1.17	5.94	246.4
12	1-Pentyloctyl benzene	1.189	5.98	260.5
13	1-Butylnonyl benzene	1.196	4.17	260.5
14	1-Propyldecyl benzene	1.21	4.13	260.5
15	1-Ethylundecyl benzene	1.22	2.30	260.5
16	1-Methyldodecyl benzene	1.267	3.51	260.46
17	Phytol	1.44	0.16	128.17
18	Heneicosane	1.68	0.2	296.6
19	Squalene	1.69	3.32	410.7
20	Nerolidol	1.76	5.13	222.3
21	cis-Farnesol	1.8	4.08	222.37
22	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]trimethylsilyl]oxy]-17-[O-(phenylmethyl)oxime]	1.92	3.62	481.75
23	Pentatriacontane	1.96	0.6	492.9
24	Stigmastan-6,22-dien-3,5-dedihydro	2.11	0.02	394.67
25	4,6-Cholestadienol	2.136	0.02	384.6
26	Stigmastan-3,5-diene	2.15	2.44	396.7
27	Pregnan-20-one,3-(acetyloxy)-5,6:16,17diepoxy	2.2	2.79	388
28	β-Sitosterol	2.3	3.32	414.7

RRt = relative retention time to 1-propyloctylbenzene.

represented by 3 compounds (12.53%), squalene 3.32%, nerolidol 5.13%, and cis-Farnesol 4.08% as shown in Table 4.

Cytotoxic activity

This study reported the *in-vitro* cytotoxicity evaluations of the *D. spinulosum* extracts by sulforhodamine B assay against MCF-7 and HeLa cell lines; *D. spinulosum* showed good cytotoxic

activity against HeLa cell line, as the total ethanolic extract, *n*-hexane, DCM, ethyl acetate, and *n*-butanol fractions showed IC₅₀ of 21.8, 21.1, 24.5, 26.5, and 21.4 µg/ml, respectively. The total ethanolic extract, EtOAc fraction of *D. spinulosum*, and aromadendrin (separated from EtOAc fraction) exhibited good cytotoxic activity against MCF7 cell line (IC₅₀ of 22.5, 18, and 12.5 µg/ml, resp.), as shown in Table 5 and Figure 2.

Table 5. Cytotoxic potential of different extracts of *D. spinulosum* leaves against endocervix carcinoma (HeLa) and breast cancer (MCF7) cell lines.

Cell line	IC ₅₀ (µg/ml)					
	Total ethanolic extract	n-Hexane fraction	Methylene chloride fraction	Ethyl acetate fraction	n-Butanol fraction	Aromadendrin
MCF7	22.5	NA	NA	18	NA	12.5
HeLa	21.8	21.1	24.5	26.6	21.4	NA

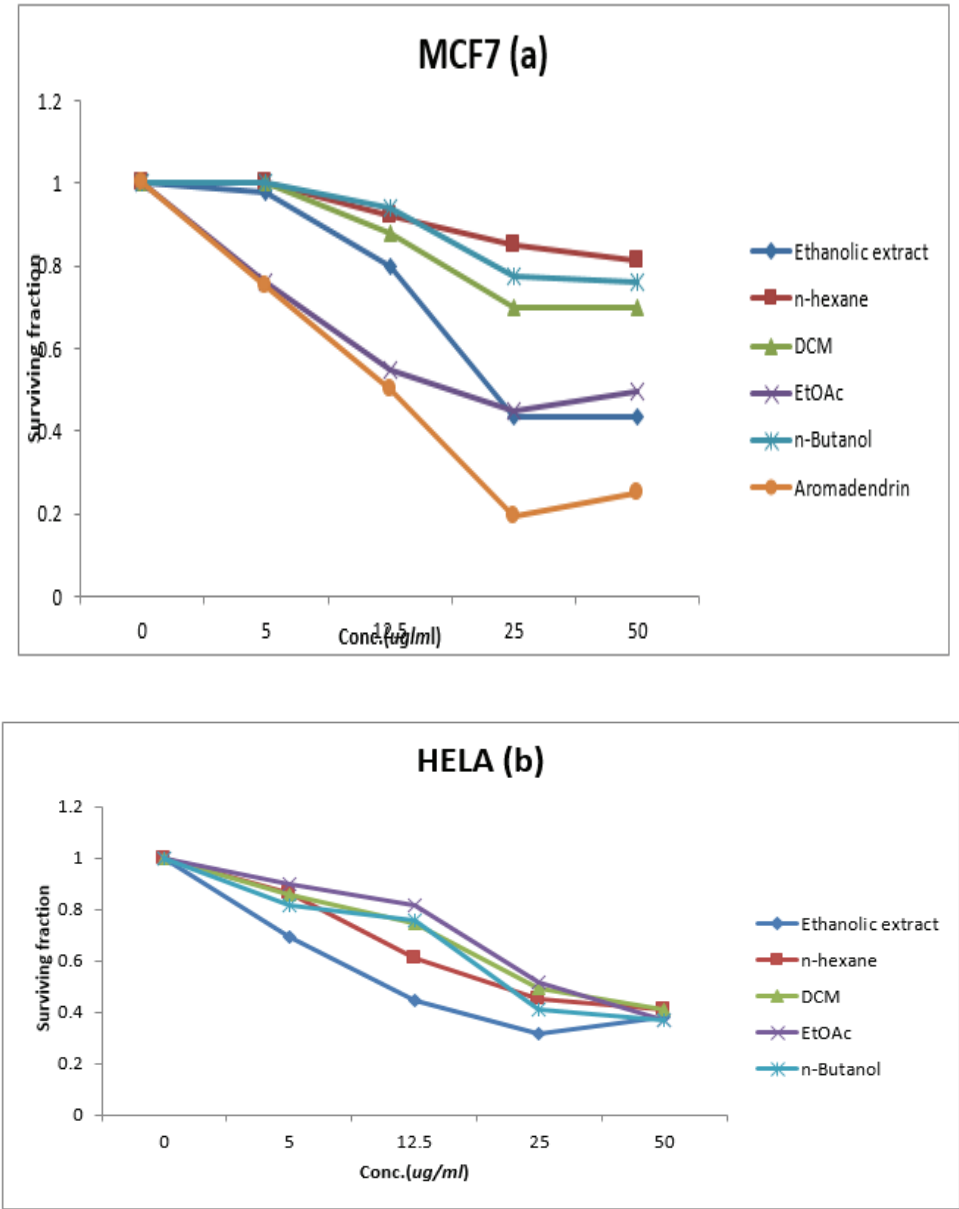


Figure 2. Cytotoxic effect of *D. spinulosum* leaf extracts against breast cancer (MCF-7) cell line (a) and human endocervix carcinoma (HeLa) cell lines (b).

DISCUSSION

Dioon genus can be differentiated from other cycad genera by the lack of midribs in their leaflets. There is a close similarity between *Dioon* species in morphological characters. Molecular markers have a higher precision in genetic differentiation and diversity studies (Satya *et al.*, 2015). Of the various DNA marker systems, the effectiveness of the SCoT markers for identification of the species and assessing its genetic diversity (Collard and Mackill, 2009) is superior to other DNA marker systems, like Random Amplified Polymorphic DNA (RAPD) and ISSR for higher polymorphism (Gorji *et al.*, 2011). The DNA analysis of two *Dioon* species, *D. edule* and *D. spinulosum*, showed a total polymorphism of 33.33% using the ISSR markers and a total polymorphism of 45.16% using the SCoT markers, which indicates the genetic diversity of the two species.

Chromatographic analysis and isolation of major active constituents revealed that *D. spinulosum* leaves contain many secondary metabolites with previously reported anticancer activity.

Amentoflavone is one of the biflavonoids existing in all cycads and is considered as a chemical marker in these plants. It is a major compound in *D. spinulosum* leaves, as previously reported by Meurer and Stevenson (1994). Amentoflavone showed potent cytotoxicity against a variety of human cancer cell lines, i.e., glioblastoma (Ibrahim *et al.*, 2006), melanoma (Guruvayoorappan and Kuttan, 2007), cervical carcinoma (Lee *et al.*, 2011), breast and endocervix (Lee *et al.*, 2012), erythroleukemia and nasopharyngeal carcinoma (Li *et al.*, 2014), and lung adenocarcinoma (Jung *et al.*, 2017).

β -Sitosterol has a history of anticancer activity against many human cancer cell lines: larynx carcinoma (Matos *et al.*, 2006), breast carcinoma (Awad *et al.*, 2007), and colon carcinoma (Baskar *et al.*, 2010). Furthermore, 7,7'',4',4'''-O-tetra-methyl amentoflavone showed good cytotoxic activity against lung adenocarcinoma cell line (PC-9) (Li *et al.*, 2014). Generally, biflavonoids isolated from many plants reported good cytotoxic activity, for example, biflavonoids from *Selaginella delicatula* (Lin *et al.*, 2000) and *Anacardium occidentale* L. (Konan *et al.*, 2012).

Aromadendrin has reported cytotoxic activity against breast carcinoma (BT474), lung bronchus carcinoma, liver carcinoma, gastric carcinoma, colon adenocarcinoma (Chunhakant and Chaicharoengpong, 2019), and ovarian carcinoma (Kwak *et al.*, 2009).

GC-MS analysis of the USM revealed the presence of other sterols and terpenes that may contribute to the cytotoxic activity of *D. spinulosum*. A large number of hydrocarbons (18 compounds identified in GC-MS, representing 75.02% of the unsaponifiable matter, may be attributed to foliar epicuticular wax predominant in cycads (Osborne *et al.*, 1993).

From the identified secondary metabolites and their previously documented cytotoxic activity, we can report that the presence of biflavonoids, β -sitosterol, and aromadendrin contributed to the cytotoxic properties of *D. spinulosum* leaves against breast and endocervix carcinoma cell lines. Thus, the plant under investigation offers a potential herbal product for adjunctive therapy in cancer patients.

CONCLUSION

DNA fingerprinting of *D. spinulosum* Dyer ex. was determined by SCoT 4, SCoT 6, SCoT 8, and HB 9 since they generated the largest number of bands with large molecular sizes. The phytochemical examination resulted in the isolation of five compounds from *D. spinulosum*, including one sterol, three biflavones, and one flavanone. β -Sitosterol and aromadendrin were obtained for the first time from this plant. The assessment of *in-vitro* cytotoxic activities of the ethanolic extract and the different fractions against endocervix carcinoma (HeLa) cell line and breast cancer (MCF7) cell line showed good cytotoxic activity, and aromadendrin (separated from EtOAc fraction) is responsible for the good effect against MCF7 cell line ($IC_{50} = 20 \mu\text{g/ml}$).

CONFLICT OF INTEREST

All the authors declare that they have no conflicts of interest for this work.

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