Saudi Journal of Biological Sciences 28 (2021) 6324–6331

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Phytochemical profile and antiproliferative activities of acetone extracts of *Asplenium polypodioides* Blume. and *A. dalhousiae* Hook. in MDA-MB-231 breast cancer cells

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ARTICLE INFO

Article history:
Received 5 May 2021
Revised 8 June 2021
Accepted 29 June 2021
Available online 9 July 2021

Keywords:
Asplenium dalhousia
Asplenium polypodioide
MDA-MB-231 cells
Anticancer
Antioxidant

ABSTRACT

The plants extracts are widely used in traditional medicines and hence considered a potential source for drug discovery. In this study, we assessed the phytochemical composition of *Asplenium dalhousiae* and *Asplenium polypodioides* in acetone extracts and checked its antiproliferative potential in MDA-MB-231 cells. We found that both plants are rich in phenolic and flavonoid compounds and are efficient in antioxidant activities. The total phenolic compounds in *A. dalhousiae* were 44.15 ± 1.38 μg/mg whereas in *A. polypodioides* were 27.73 ± 1.35 μg/mg. Total flavonoids in *A. dalhousiae* were 105.39 ± 2.92 μg/mg whereas in *A. polypodioides* were 101.56 ± 1.75 μg/mg. The ferric reducing power assay indicates 66.38 ± 2.6% reduction by *A. dalhousiae* whereas 78.43 ± 0.47% reduction by *A. polypodioides.* Similarly, the total antioxidant capacity of *A. dalhousiae* was found to be 59.95 ± 1.13 whereas for *A. polypodioides* the recorded value was 33.03 ± 1.67%. Using GCMS analysis, we identified 25 compounds in *A. dalhousiae* whereas 26 in *A. polypodioides.* Four of these compounds are common in both plants. The morphological study and MTT assay revealed that both plants have antiproliferative potential as both plants exerted significant effects on the shape of the MDA-MB-231 cells and inhibited cellular proliferation in time and dose dependent manner. We conclude that both *Asplenium* plants have potential anticancer compounds.

1. Introduction

Plants are considered as a good source of chemicals that can be used to treat different diseases including cancer. These plant derived compounds are considered as safer, eco-friendly, cost effective and less toxic compared to existing treatment methods (Wang et al., 2012; Iqbal et al., 2017). About 50–60% of cancer patients in the United States rely on plant-derived agents as an alternative therapy and can be administered alone or simultaneously with other therapies such as chemo or radiotherapy (Gutheil et al., 2012). The overall use of natural products in the United States has increased within the last few years. In 2007, it was reported that 18% of American adults have been using natural products beyond a basic multivitamin (Greenlee, 2012).

Genus *Asplenium* consists of around 700 species of ferns and belongs to the family "Aspleniaceae". These species are widely spread around different areas in Pakistan (Lower Dir, Upper Dir, Chitral, Shangla, Alpuri, Shahpur, Lilownia, Chakesar, Swat, Murree, Ajaori, Kashmir, and Kass) (Zaman et al., 2019). Many *Asplenium* species such as *A. Nidus* (Jarial et al., 2018), *A. dalhousiae* (Ullah et al., 2018), *A. africanium* (Ondo et al., 2013), *A. ruprechtii* (Wang et al., 2020) are proven source of anti-cancer and antiangiogenic phytochemicals (Abbaszadeh et al., 2019).

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.06.098
1319-562X/

According to estimates from World Health Organization, cancer was the leading cause of death for people under the age of 70 in 112 out of 183 countries (Sung et al., 2021). In the United States alone, the estimated number of cancer incidence was 1,806,590 and estimated number of deaths due to cancer was 606,520 in 2020 (Siegel et al., 2020). Among cancers, breast cancer stands second most common reason to cancer related deaths (Uzma et al., 2020), that makes its place in the three most common cancers worldwide (Harbeck and Gnant, 2017). In the Middle East and Gulf Region, the incidence rates of breast cancer are rising drastically and affecting a younger population at higher rates compared to Western countries (Radi, 2013).

Furthermore, the number of breast cancer cases is estimated to be doubled by 2030 in the Middle East (Rizvi, 2016; Suchitra Bajpai Chaudhary, 2018). In United Arab Emirates (UAE), breast cancer has the highest prevalence followed by colorectal and lung cancers (Rahman et al., 2019). There are three main subtypes of breast cancer depending on (i) the presence or absence of receptors for progesterone or estrogen, (ii) human epidermal growth factor 2 (*ERBB2*; formerly *HER2*) receptor and (iii) triple negative breast cancer (TNBC) (where all the three receptors are absent). TNBC accounts for 15% of patients, whereas hormone receptor positive/*ERBB2* negative accounts for 70% of patients and *ERBB2* positive for 15–20%. The TNBC has higher recurrence rates compared to the other two subtypes. Patients suffering from TNBC stage I show 10% lower survival rate compared to the to hormone receptor positive and ERBB2 positive cancers (Waks and Winer, 2019). MDA-MB-231 cell line is used as an in-vitro model for the TNBC (Khattak et al., 2020: AlGhalban et al., 2021). In this context, the current study was designed to know the phytochemical composition of the acetone extracts of the two *Asplenium* species i.e., *A. dalhousiae* and *A. polypodioides*. Furthermore, the antiproliferative and cytotoxic effects of the same was studied in TNBC in vitro model MDA-MB-231 breast cancer cell line.

2. Materials and methods

2.1. Collection of plant material, identification and preparation of extracts

A. polypodioides and *A. dalhousiae* plants were collected from different localities of Rawlakot, District Poonch, Kashmir and identified at department of Botany, Pir Mehr Ali Shah (PMAS), Arid Agriculture University, Rawalpindi, Pakistan by Dr Rahmatullah Qureshi, and the Voucher specimens of the identified species were submitted to the Herbarium in the same department. The plant material was washed to remove any unwanted fibers and dirt particles. Then it was dried in shade at room temperature and finely powdered with an electric mill. The powder was stored in different sealed bags for further analysis. Plant sample (2 g) of each plant was extracted in 25 ml acetone. It was shaken at room temperature using rotary shaker (HZ-300 rotary shaker) for 24 h followed by filtration. The solvent from the filtrate was evaporated at 45 °C using rotary evaporator and the extract were recovered in the form of crude powder, added to pre-weighed Eppendorf tubes.

For further analysis, the stock concentration (1 mg/ml) was prepared by dissolving the crude extracts in 1% dimethyl sulfoxide (DMSO) or in sterile nuclease free water and then stored at 4 °C till further use.

2.2. Quantitative analysis of phytochemicals

2.2.1. Determination of total phenolic contents

Total phenolic contents were determined by Folin-Ciocalteu (FC) reagent assay (Singleton and Rossi, 1965) with some modification for microplate analysis (Wakeel et al., 2019). Stock solution of FC reagent was prepared by diluting 10 times the FC reagent (density: 1.24 g/cm^3) with deionized distilled water. The assay was performed in a 96 well plate by adding 20 μl of the test sample and 90 μl of FC reagent. The plate was incubated for 5 min and after incubation, 90 μl of 6% sodium bicarbonate solution was added into it. The plate was further incubated at room temperature for 90 min. Gallic acid 40, 20, 10, 5 and 2.5 μg/ml final concentration was used as standard drug in separate wells of 96 well plate. Readings were noted at 630 nm wavelength on microplate ELISA reader.

2.2.2. Determination of total flavonoid contents

The total flavonoid contents were determined by following Chang et al. (2002) with slight modification for microplate reading (Wakeel et al., 2019). Briefly, stock solution of 10% $AlCl_3$ and 1 M potassium acetate were prepared separately. Various concentrations of test samples (20 μl) were taken in 96 well plate. Quercetin 50, 25, 12.5, 6.25 μg/ml final concentration was used as standard drug in separate wells of 96 well plate. To each test sample and standard drug, 10 μl of $AlCl_3$ and 10 μl potassium acetate were added. The volume of the reaction was made to 200 μl by the addition of 160 μl distilled water. The plate was incubated for 30 min and read at 405 nm wavelength on microplate ELISA reader. Standard curve was prepared using Excel trendline curve and the amount of total flavonoid was expressed as Quercetin equivalent μg/mg of extract.

2.3. Antioxidative activity assays

2.3.1. DPPH assay

Free radical scavenging activities were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent which contains stable free radical molecules (Brand-Williams et al., 1995) with modification for microplate analysis (Wakeel et al., 2019). Stock solution was prepared by dissolving 9.6 mg of DPPH in 100 ml methanol and the optical density was adjusted to 1 through spectrophotometer. The assay was performed by adding 20 μl of test sample and 180 μl of DPHH solution in 96 well plate. Ascorbic acid was used as positive control in the assay as a standard free radical scavenger. The plate was incubated for 1 h and read at 517 nm wavelength on microplate ELISA reader. Percent scavenging activities was calculated using the following formula:

$$\% \text{ scavenging} = \left[1 - \left(\frac{\text{OD of test sample}}{\text{OD of Control}}\right)\right] \times 100$$

A graph of inhibition percentages against concentration of the sample using trend line (y = ax + b) was plotted to calculate 50% inhibition (IC_{50}) values.

2.3.2. Total antioxidant capacity (Phosphomolybdenum method)

Total antioxidant capacity of the sample was determined by Phosphomolybdenum assay (Jafri et al., 2017; Mehwish et al., 2019). The stock solution of Phosphomolybdenum reagent was prepared by dissolving 1.63 ml of sulphuric acid (0.6 M), 1.6795 g NaH_2PO_4 (28 mM) and 0.247 g of Ammonium molybdate (4 mM) in 50 ml distilled water in a reagent bottle. The assay was performed in 1.5 ml Eppendorf tube by adding 20 μl test sample and 180 μl of the reagent. The mixture was incubated at 95°C for 90 min in water bath. The mixture was cooled into room temperature and transferred to 96 well plate for reading at 630 nm wavelength on microplate ELISA reader. Ascorbic acid standard drug was used as positive control. Percent antioxidant capacity was calculated as:

$$\% \text{ antioxidant capacity} = \left[1 - \left(\frac{\text{OD of test sample}}{\text{OD of Control}}\right)\right] \times 100$$

2.3.3. Ferric reducing capacity assay

The ferric reducing capacity of extracts was investigated by using the potassium ferricyanide-ferric chloride method (Benzie and Strain, 1996) with some modification (Wakeel et al., 2019). Briefly, test sample of 40 μl was poured into 1.5 ml Eppondorf tube. 50 μl phosphate buffer (0.2 M, pH 6.6) and 50 μl potassium ferricyanide (1%) were added into it and incubated at 50 °C for 20 min. Then 50 μl of 10% Trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer 166.66 μl was transferred to another tube and 33.3 μl of 0.1% Ferric chloride was added. The same procedure was adopted for the standard drug ascorbic acid. The tube was vortexed and transferred the mixture to 96 well plate for reading at 630 nm wavelength on microplate ELISA reader. Percent reducing power was calculated using th following formula:

$$\%\ \text{reducing power} = \left[1 - \left(\frac{\text{OD of test sample}}{\text{OD of Control}}\right)\right] \times 100$$

2.4. Gas chromatography-mass spectrometry (GC–MS)

To identify the chemical composition of acetone extracts of *A. polypodioides* and *A. dalhousiae*, GC–MS analysis was performed using a GC–MS QP 2010 ultra, Shimadzu-Japan. GC separation was conducted using a general-purpose nonpolar crossband dimethylpolysiloxane RTX-1 Column from RESTEK Company. The column is 30 m long with 0.25 mm internal diameter. The temperature program setup was as follows: 2 min at 80 °C, then the temperature ramped to 300 °C with heating rate of 5 °C/min and held at 300 °C for 10 min. The temperature of the injector was 280 °C. Helium was used as the carrier gas with a flow rate of 0.9 ml/min. The corresponding mass spectrum for each identified GC peak was analyzed based on GCMS solution Wiley-9 and NIST-14 libraries with 90% match and above.

2.5. Anticancer activities

2.5.1. Cell culture and treatments

Human triple negative breast cancer cell lines MDA-MB-231 were cultured in 96-well plates with 7,000 cells per well using liquid Dulbecco's Modified Eagle Medium (DMEM) High Glucose (Sigma) where we added 10% Fetal Bovine Serum (Sigma) and 1% Penicillin and Streptomycin (Gibco™). The cells were incubated for 24 h at 37 °C and 5% CO_2 in 95% humidity. The cells were treated with 4 different concentrations using the following experimental design:

Group 1: Untreated control, where the medium was changed after every 24 h and reading was noted each at 24, 48 and 72 h.

Group 2: After 24 h of seeding, the cells were treated with 50, 100, 200, 300 μg/ml concentrations of *A. polypodioides* or *A. dalhousiae* extracts and readings were noted after 24 h.

Group 3: After 24 h of seeding, the cells were treated with 50, 100, 200 and 300 μg/ml concentration of *A. polypodioides* or *A. dalhousiae* extracts for 48 h (24 h + 24 h) and the readings were noted at 48 h.

Group 4: After 24 h of seeding, the cells were treated with 50, 100, 200, 300 μg/ml concentrations of *A. polypodioides* or *A. dalhousiae* extracts for 72 h (24 h + 24 h + 24 h) and the readings were noted at 72 h.

2.5.2. Morphological study

To observe the effects of the acetone extract on shape and adherence of the MDA-MB-231 cells, cells in 96-well plates were observed under a phase contrast microscope (OPTIKA, Italy) using 40X magnifications and photographed for all time points mentioned in the above experimental design. The cells were photographed under the inverted microscope using Optika vision lite software. Pictures were saved in JPG format for further study and analysis.

2.5.3. Methyl Tetrazolium-MTT assay

MTT assay was performed at 24, 48 and 72 h according to the experimental design mentioned in the section 2.5.1. For MTT assay, the medium was removed from each well and 200 μl (40 μl of MTT reagent + 160 μl of Medium) of working MTT solution was added and incubated for 4 h at 37 °C. The working reagent was removed gently and 200 μl of DMSO was added to each well and was shaken gently for 15 min at room temperature in dim light. The color of the wells turned violet and was read at 570 nm wavelength using ELISA microplate reader (Thermo Scientific™ Multiskan™ GO UV/Vis microplate spectrophotometer, USA). All the experiments were performed three times in triplicate and data are expressed as Mean ± SD.

2.6. Statistical analysis

All the experiments were performed three times in triplicate. Cell viability percentage was calculated using the formula: [(Average Sum of the triplicates ODs)/ (corrected OD of control) × 100]. After calculating percent means of all the three experiments, the standard deviation was calculated. The statistical significance of the differences between experimental variables and their reference group was determined using the One-way ANOVA and Tukey's HSD *t* test. $P < 0.05$ was considered statistically significant. The values shown represent the mean ± SD.

3. Results

3.1. Quantification of total phenolic contents

Table 1 shows the total phenolic contents (TPC) of acetone extracts in microgram (μg) Gallic acid equivalent (GAE) per milligram (mg) of extracts. The results were quantified through gallic acid standard curve using trendline at y = 0.0268x + 0.0753 and linear regression at R^2 = 0.9986. The results show that phenolic contents of the acetone extract of *A. polypodioides* has 27.73 ± 1.35 μg GAE/mg whereas *A. dalhousiae* is 44.15 ± 1.38 μg GAE/mg.

3.2. Quantification of total flavonoid content

The total flavonoid contents in *A. polypodioides* and *A. dalhousiae* extracts were quantified through microplate method using Quercetin standard calibration curve at y = 0.0136x + 0.0845 and linear regression at R^2 = 0.9861. Table 1 shows the total flavonoid contents (TFC) of acetone extracts expressed as μg Quercetin equivalent (QE)/mg of extracts. The highest number of flavonoid contents was quantified in the acetone extract of *A. polypodioides* as 101.56 ± 1.75 μg QE/mg. Similarly, the amount of TFC quantified in acetone extract of *A. dalhousiae* was 105.39 ± 2.92 μg QE/mg (Table 1).

Table 1
Quantitative analysis of total phenolic contents (TPC), total flavonoid content (TFC), ferric reducing power assay (FRPA) and total antioxidant capacity assay (TAC) in Acetone extracts at final concentration of 1 mg/ml.

	A. *polypodioides*	*A. dalhousiae*
TPC	GAE = 27.73 ± 1.35 μg/mg extract	GAE = 44.15 ± 1.38 μg/mg extract
TFC	QE = 101.56 ± 1.75 μg/mg extract	QE = 105.39 ± 2.92 μg/mg extract
FRPA	% age reduction = 78.43 ± 0.47	% age reduction = 66.38 ± 2.6
TAC	% age TAC = 33.03 ± 1.67	% age TAC = 59.95 ± 1.13

3.3. Antioxidative activities of the extracts

3.3.1. DPPH free radical inhibition

Free radical inhibition was estimated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a source of free radical and ascorbic acid as standard free radical inhibitor. The percentage of inhibition by various concentration (25, 50, 100 and 200 μg/ml) of acetone extracts was estimated in the extracts of both the plants *A. polypodioides* and *A. dalhousiae* as shown in table 2. It has been observed that there as a direct relationship of the concentration of extracts and the inhibition of the free radical in the reaction mixture. The IC_{50} values were calculated through linear trend line analysis (y = ax + b) of the concentration versus percent inhibition. It was observed that the extract of *A. polypodioides* in acetone require low IC_{50} concentration (36.8 μg/ml) compared to *A. dalhousiae* (16.833 μg/ml). This indicates that each plant extract has different efficiency in extracting phytochemicals that have differential inhibition activities (Table 2).

3.3.2. Ferric reducing power assay (FRPA)

The reduction of ferricyanide (Fe^{3+}) to ferrous state (Fe^{2+}) by the plant phytochemical is a good indication of antioxidant activity (Ojo et al., 2017). In the current study, the percentage (%) of reducing power of the acetone extract of *A. polypodioides and A. dalhousiae* was estimated against standard drug ascorbic acid (Table 1). The reduction capacity of *A. polypodioides* acetone extract was 78.43 ± 0.47% and *A. dalhousiae* acetone extract was 66.38 ± 2.6%.

3.3.3. Total antioxidant capacity (TAC)

Total antioxidant capacity was determined by the Phosphomolybdenum assay spectrophotometrically using ELISA Plate reader (Prieto et al., 1999). Table 1 indicates the percentage capacity of the extracts of *A. polypodioides* and *A. dalhousiae*. The percentage of TAC for the acetone extracts of *A. polypodioides* was 33.03 ± 1.67 and for *A. dalhousiae* it was 59.95 ± 1.13% in acetone extract.

3.4. Gas Chromatography–Mass Spectrometry (GC–MS) analysis

We rely on GC–MS spectrometry to identify the major components in both *A. polypodioides* and *A. dalhousiae* extracts. Fig. 1 shows the GC chromatogram of *A. polypodioides* and *A. dalhousiae* acetone extracts. As indicated in Fig. 1, there are various common compounds identified in both extracts with higher population observed in *A. polypodioides* sample in most cases. Table 3, summarizes the major common compounds identified in both extracts. Beside the major compounds observed in both extracts, several weak GC peaks observed in a given extract and mainly characterized as aliphatic and aromatic hydrocarbons. For example, in *A. polypodioides* extract, trace amount of 1-dodecanol (21.7 min), 2-pentanone, 4-hydroxy-4-methyl (6.8 min), 1,2,3-propanetriol, and quinic acid (22.95 min). In contrast, various aromatic compounds like Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy

Table 2
Percent (%) free radical inhibition activities using DPPH as free radical source.

Final Conc. (μg/ml)	*A.polypodioides* Blume (Extract) Acetone	*A.dalhousiae* Hook (Extract) Acetone
200	83.87%	44.62%
100	69.30%	38.78%
50	59.01%	37.28%
25	39.07%	27.80%
IC_{50} Value (μg/ml)	36.8	16.33

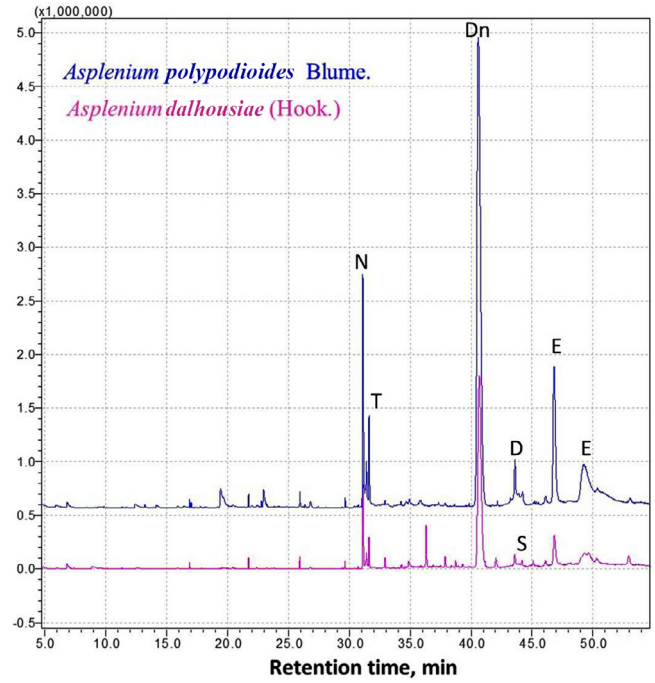


Fig. 1. GC-MS chromatogram of *A. polypodioides* and *A. dalhousiae* acetone extracts.

(32.95 min), 4,8,12,16-Tetramethylheptadecan-4-olide (37.9 min), and 4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dimethoxy-2-phenyl (38.7 min) were identified in *A. dalhousiae* extract. Squalene, a molecule that showed anti-cancer and antioxidant activities in previous study (Kim and Karadeniz, 2012) was also identified with a GC peak appear after 44.2 min.

3.5. Effects of A. polypodioides and A. dalhousiae acetone extract on morphology of MDA-MB-231 cells

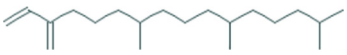
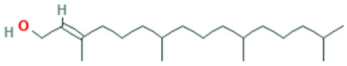
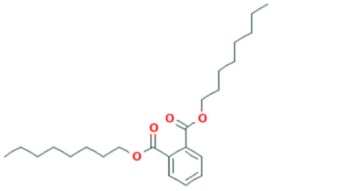
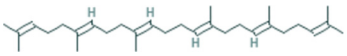
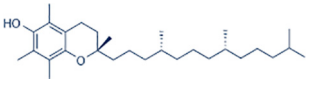
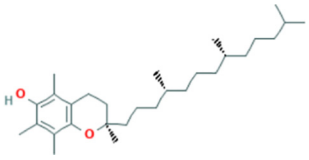
The acetone extracts of *A. polypodioides* caused a time (24, 48 and 72 h) and dose (50, 100, 200 and 300 μg/ml) dependent change in the morphology of the cells. As shown in Fig. 2, the untreated MDA-MB-231 cells maintained their cohesion and original epithelial shape whereas in treated wells, the cells lost their epithelial shape and more dead cells were observed with increase in time and concentration. As evident in the Fig. 2, at 72 h, with treatment dose of 300 μg/ml of the extract, the cells have lost their adherence and became spheroidal (rounded) shaped. Moreover, the space between the cells have increased significantly with the increase in concentration, indicating reduction in number of cells (Fig. 2).

The acetone extracts of *A. dalhousiae* also exerted its effects in time and dose dependent manner. The untreated MDA-MB-231 cells maintained its original cell morphology, whereas the treated cells clearly showed a change in the cell morphology However, when comparing the cells density between control and treated, it decreased by half at time points using the 100, 200 and 300 μg/ml. This is a clear sign that this extract has dropped the cell proliferation rate drastically. On the other hand, as shown in Fig. 3 the cells have lost their original epithelial shape at the lowest used concentration (50 μg/ml) for the three time points indicated (Fig. 3).

3.6. Effects of A. polypodioides and A. dalhousiae acetone extract on cell viability of MDA-MB-231 cells.

Our results show that treatment of MDA-MB-231 cell line with different concentrations of the acetone extract of *Asplenium* plants

Table 3
List of common compounds found in both *Asplenium polypodioides* and *A. dalhousiae* extracts as identified using GC–MS spectrometry.

Retention time (min)	Compound name	Molecular formula	Structure	Peak Label	MW (Base Peak)	Area/ Height
31.1	Neophytadiene	$C_{20}H_{38}$		N	278 (95)	7.58
31.4	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$		T	296	6.18
40.7	Di-n-octyl phthalate	$C_{24}H_{38}O_4$		Dn	390 9149)	17.78
43.6	Decanedioic acid, bis(2-ethylhexyl) ester	$C_{26}H_{50}O_4$		D	426	6.41
44.2	Squalene	$C_{30}H_{50}$		S	410 (149)	6.17
46.8	Vitamin E (isomers)	$C_{29}H_{50}O_2$		E	430 (165)	10.97
49.7	Vitamin E (isomers)	$C_{29}H_{50}O_2$		E	430 (165)	9.87

*Various conformations.

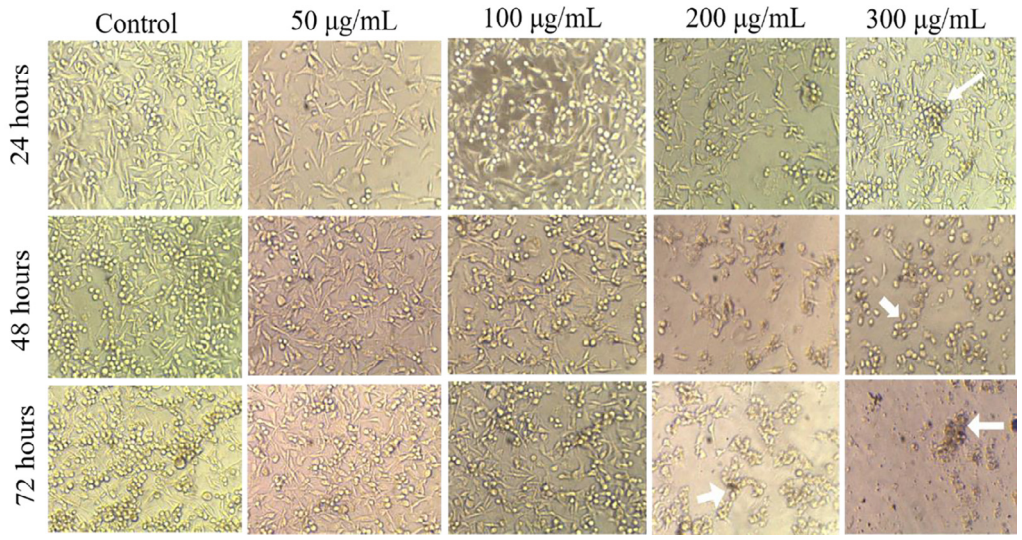


Fig. 2. Morphological changes in MDA-MB-231 breast cancer cells after treatment with four different concentrations (50, 100, 200 and 300 µg/ml) for three times points (24, 48 and 72 h) with acetone extracts *A. polypodioides*. The white arrows pointed at the suspension of dead cells.

decreased cellular viability in concentration and time dependent manners. In general, we found that the total cell proliferation decreased compared to the untreated control.

At 24 h, the cell viability was significantly ($p < 0.05$) reduced by 60% each in 50, 100 and 200 µg/ml using acetone extracts of *A. polypodioides* compared to untreated control, whereas treatment with 300 µg/ml caused 95% reduction compared to untreated control. Furthermore, at 48 h, the cell viability further significantly ($p < 0.05$) reduced by 70% in 50 µg/ml and 200 µg/ml treated groups, whereas in 100 µg/ml treated group it remained at the same level as in 24 h. In the group treated with 300 µg/ml, the reduction was maintained at 95%. At 72 h, 50, 100, 200 and 300 µg/ml concentration of acetone extracts of *A. polypodioides* caused reduction in cell viability by 40%, 50%, 70% and 95% respectively (Fig. 4).

The cell viability at 24 h was remarkably decreased by 60% in acetone extracts of *A. dalhousiae* in each 50, 100 and 200 µg/ml concentrations compared to the untreated control. However, treat-

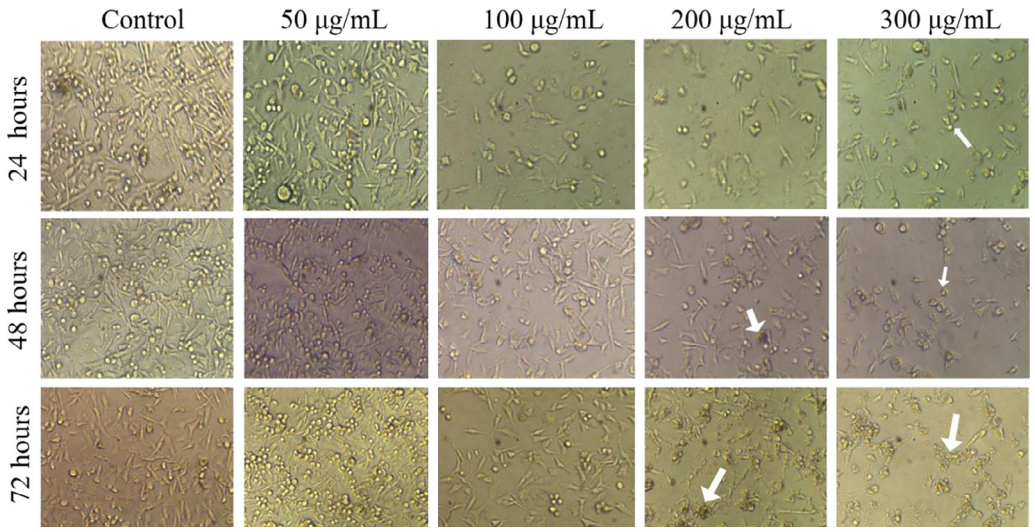


Fig. 3. Morphological changes in MDA-MB-231 breast cancer cells after treatment with four different concentrations (50, 100, 200 and 300 µg/ml) for three times points (24, 48 and 72 h) with acetone extracts *A. dalhousiae*. The white arrows pointed at the suspension of dead cells.

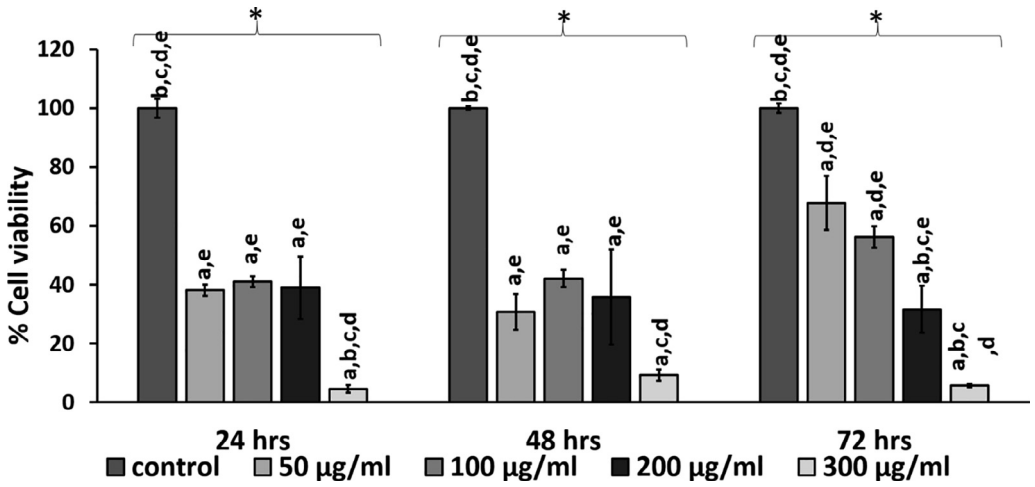


Fig. 4. Effect of acetone extracts of *A. polypodioides* on viability of MDA-MB-231. The cells were treated with four different concentrations (50, 100, 200 and 300 µg/ml) for three times points (24, 48 and 72 h). The cells viability is affected in dose and time dependent manner. Symbols indicates significant difference between different treatments: (a) untreated control, (b) 50 µg/ml (c) 100 µg/ml (d) 200 µg/ml (e) 300 µg/ml. * shows $p < 0.05$ for ANOVA comparing all the groups.

ment with 300 µg/ml caused 80% reduction. Moreover, at 48 h, the cell viability was decreased by 40% in 50, 100, 300 µg/ml treated groups, whereas in 200 µg/ml the cells viability was reduced by 60% compared to the untreated control group. In addition, at 72 h, the cells viability was reduced by 40% for the treatment using 300 µg/ml (Fig. 5).

4. Discussion

Plants are a rich source of phytochemicals that are used traditionally as an ailment and many of these are still unexplored. The phytochemical composition of plants varies with species, geographical conditions, parts of the plants and even with the extraction method and solvent used for extraction. Therefore, each plant is unique and may have a potential to be used as a medicinal plant.

Phenolic compounds from plants are evident in treatment of cancer (de Oliveira Melo et al., 2018). In our study, we found phenolic contents in both plants species, however total phenolic contents were higher in *A. dalhousiae* (44.15 µg/mg) compared to A. *polypodioides* (27.73 µg/mg). Different researchers report that the phenolic contents reported are different with respect to type of solvent used, method of extraction, type of plant, part used and analysis method (Kim and Lee, 2004: Shan et al., 2005). The difference in phenolic contents in the two *Asplenium* species is not unusual as different plants are reported with different phenolic contents (Dorman et al., 2003: Chan et al., 2008: Wongsa et al., 2012). Flavonoids are one of the three major secondary metabolites in the plant kingdom. They have shown anticancer activities such as, inhibition of cancer cell growth, antioxidant activity, apoptosis induction and cancer cell cytotoxicity (Greenwell and Rahman, 2015). In our study, the total flavonoids were comparable not only in both *Asplenium* plants, but with also with other plants. This indicate that both of these plants have compound, which has moderate polarity. This shows that these plants are rich source of various plant flavonoids compounds that may have potential antioxidant and biological activities in both enzymatic and non-enzymatic systems. The direct relationship between the flavonoid contents and antioxidant activities is established (Sharififar et al., 2009). Our results are supporting this by showing direct relationship of the

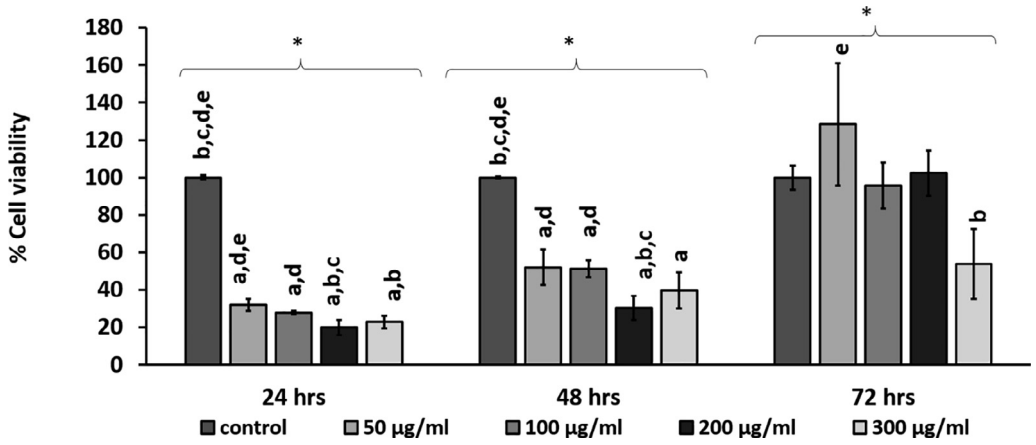


Fig. 5. Effect of acetone extracts of *A. dalhousiae* on viability of MDA-MB-231. The cells were treated with four different concentrations (50, 100, 200 and 300 μg/ml) for three times points (24, 48 and 72 h). The cells viability is affected in dose and time dependent manner. Symbols indicates significant difference between different treatments: (a) untreated control, (b) 50 μg/ml (c) 100 μg/ml (d) 200 μg/ml (e) 300 μg/ml * shows $p < 0.05$ for ANOVA comparing all the groups.

phenolic contents and flavonoids with antioxidant activities. A study conducted on *Ficus microcarpa* extracts showed, a positive correlation between the antioxidant activity and total phenolic content, whereby the correlation coefficient (R2) was 0.919, 0.836, 0.813 and 0.534 for ABTS, DPPH, PMS–NADH, b-carotene bleaching methods, respectively. The results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the *F. microcarpa* extracts (Ao et al., 2008). Another study on extracts of *Camellia sinensis* (L.) O. Kuntz (green tea), *F. bengalensis* L. and *F. racemosa* L, reported the total % phenolic contents in acetone extracts to be 47.6 ± 3.3 and 60.3 ± 4.5 and 54.8 ± 1.2 (Manian et al., 2008). In our study, the TPC of both *A. polypodioides* and *A. dalhousiae* given in GAE were 27.73 ± 1.35 and 44.15 ± 1.38 μg/mg respectively. We found that acetone extracts of both *Asplenium* plants have antioxidant activities based on DPPH assay. Our results are in close agreement to the result reported for other plants such as *F. macrocarpa* which also showed excellent antioxidant and antibacterial activities (Ao et al., 2008). It is reported that extraction methods are crucial for getting extracts with acceptable yields and strong antioxidant activity (Moure et al., 2001). Similar trend of antioxidant activities has been observed for *Litchi chinenesis* L. extract (Liu et al., 2009), *Trifolium pratense* L. (Khorasani Esmaeili et al., 2015) and other Egyptian plants (Maswada and Maswada, 2013). Furthermore, out study shows that *Asplenium* plants are efficient in reducing ferricyanide (Fe^{3+}) to ferrous state (Fe^{2+}). Our results are confirming the previous studies showing reducing power of different plant solvent extracts (Philip et al., 2011: González-Palma et al., 2016). Furthermore, the total antioxidant capacity was also checked and found both *Asplenium* plants efficient and are confirming the reports of previous studies (Katalinic et al., 2006: Proestos et al., 2013).

GC–MS analysis was performed to identify the phytochemicals present in the acetonic extract of *A. polypodioides* and *A. dalhousiae*,. We found some common chemical compounds between the acetone extracts of both *A. polypodioides* and *A. dalhousiae* plants such as Di-n-octyl phthalate, Decanedioic acid bis(2-ethylhexyl) ester, Vitamin E and Neophytadiene. Neophytadiene is a well-known antipyretic, anti-inflammatory, analgesic and anti-oxidant compound (Ruedas-Rama et al., 2012) giving both plants similar potential (see Table 3).

The plants were analyzed for their anticancer potential using MDA-MB-231 cells as models. We checked the effects of acetone extracts of both asplenium plants i.e. *A. polypodioides* and *A. dalhousiae* on viability and morphology of MDA-MB-231 cells.

Microscopic analysis showed that the MDA-MB-231 cells original shape which is categorized as being spindle-like shape changed to being rounder and that is a sign for cell cytotoxicity (Mohammed et al., 2020). Moreover, the cells exhibited uneven distribution and reduced proliferation and viability in a dose and time dependent manner. Previous research has been conducted on different species from the same genus *Asplenium* which also showed reduction in proliferation on the same cell line (Jarial et al., 2018; Mazumder et al., 2020). For further confirmation, the anti-proliferative effects of *A. polypodioides* and *A. dalhousiae* on MDA-MB-231 cells were studied using MTT assay. The cell viability test showed clear reduction in the percentage of cell viability with acetone extracts of both *A. polypodioides* and *A. dalhousiae* with all the concentrations tested at the given time points.

5. Conclusions

A. polypodioides and *A. dalhousiae* extracts in acetone have higher phenolic and flavonoid contents and have antioxidant activities. Furthermore, both have some common compounds based on GCMS and are potential candidates for anticancer activities.

Funding

This research is funded partially by office of Research and Graduate Studies, University of Sharjah through grant number 1702145049-P and partially by college of graduate studies, University of Sharjah.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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