



EVALUATION OF ANTI-CANCEROUS ACTIVITY OF *Asplenium dalhousiae* HOOK. AND *Asplenium trichomanes* L. AGAINST MCF 7 AND HCT 116 CELL LINES

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ABSTRACT

Asplenium dalhousiae Hook. and *Asplenium trichomanes* L. are used in traditional medicine, but insufficient data is available on their anticancerous activity. The extracts of both ferns were tested against human cancer cell lines viz. MCF 7 (breast carcinoma) and HCT 116 (Human colorectal carcinoma). The activity was analyzed employing MTT assay to assess growth inhibition and cell viability percentages. This method measures the reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) by mitochondrial succinate dehydrogenase to form dark blue formazan which is largely specific to a viable cell and can be extracted with an organic solvent and then quantified by spectrophotometry. Results were compared with standard anticancerous drug doxorubicin. The results thus obtained showed selective anticancerous activity. Species-specific effect was observed, ethanolic fronds extract of *Asplenium trichomanes* L. displayed strongest activity against both cell line with $90.12 \pm 0.31\%$ growth inhibition against HCT 116 and $83 \pm 0.31\%$ against MCF 7 cell line. Extracts of *A. trichomanes* performed better as compared to *A. dalhousiae*. Both species appeared to be potent inducers of necrotic cell death.

Keywords: Anticancerous, antiproliferative, growth inhibition, *Asplenium trichomanes* L., *Asplenium dalhousiae*, MCF 7, HCT 116.

1. INTRODUCTION

Even though synthetic organic chemistry advanced significantly in the 20th century, more than 25% of medicines that are licensed in developed nations come from plants directly or indirectly [1]. Secondary metabolites responsible for plant's metabolic processes and defense mechanisms are of great interest and are in high demand [2]. Pteridophytes are used by physicians in the biomedical system, Ayurvedic system, the Unani system of medicine and traditional Chinese medicinal system. These are primitive vascular plants (also known as vascular cryptogams) that primarily produce spores. Moist and shaded environments are ideal for their lush development. Pteridophytes are classified into two broad groups: lycopods and ferns. Ferns and their relatives are one of the oldest main divisions of the Pteridophyta, with over 12,000 species scattered across 250 genera [3]. Forty

taxas belonging to nineteen genera and ten families of ferns are represented in KP, Pakistan. Members of family *Pteridaceae* represents 30% taxa which is followed by *Athyriaceae* and *Dryopteridaceae* that covers another 30% while 12.5% diversity is presented by members of family *Thelypteridaceae*. The common genera presented in KP Pakistan are *Equisetum*, *Adiantum*, *Asplenium*, *Diplazium*, *Aleuritopteris*, *Pteris*, *Athyrium*, *Oeosporangium*, *Pseudophegopteria* and *Polystichum*. The members of these taxas are commonly used to treat respiratory disorders including bronchitis, asthma, pneumonia, stomach and urinary tract ailments [4]. *Asplenium* is one of the important genera of ferns presented in KP Pakistan. Members of this genera are employed in traditional medicine due to their potential therapeutic benefits. *Asplenium dalhousiae* and *Asplenium trichomanes* are important representatives of this genera.

Asplenium dalhousiae Hook. Is a small fern that grows up to 15-25 cm and have rosette arrangement of fronds. It typically has 64 spores per sporangium which are pale brown in color. It grows at elevation of 800-5000 m in Malam Jabba on rocky slopes with underground scaly rhizome which is 2-4 mm in length. It is commonly used by local people against snakebites, eczema and as vermifuge. In Typhoid, the local people use it as decoction. *Asplenium trichomanes* L. is another member of genus *Asplenium*. It has chromosomal count of 72 and grows up to 30 cm. The pinna typically has 4-8 sori that are part of evergreen fronds. It commonly grows on forest edges, walls, crevices and slopes [5]. It is widely distributed in swat valley at 5160 m elevation being common in Islampur, Utror, Gabral, Kalam and Malam Jabba. Fronds are laxative and also used to treat breast infections, cold, cough and chest congestions.

Plants have long been believed to be a rich source of anti-cancer agents. Over 60% of the anti-cancer drugs currently in clinical use are of natural origin. A selection of plants has been shown in studies to be able to induce cell death in cancers. This gives scientists the opportunity to isolate the compound responsible for this and then develop it as a possible drug. A large advantage of using plant extracts as a form of treatment for cancer is their lowered toxicity in comparison to conventional treatments such as radiotherapy and chemotherapy. This is largely due to the relative toughness of plant cells compared to mammalian cells, and the ability for the plants to have chemical defense mechanisms against herbivores and pathogens. The extract may be consumed as a foodstuff or used as a medicinal tonic, although it is likely that in some cases the active compound may be necessary by itself as a drug to ensure adequate potency [6].

Cancer is the second most common cause of death in the developed world, with 67% of the population supposed to be affected by it some time during their life. It is expected that as the population ages, there will be more people diagnosed with the disease, making the burden on both the patient and the health service even greater. It is estimated that annual cancer cases will rise from 14 million in 2012 to 22 million within the next two decades. Whereas the rise in cancer incidence is increasing, the death rate is declining. This is due to earlier diagnosis and better treatment. The treatment of cancer has improved over the years, but it is still inadequate. With an improved understanding of the mechanisms of carcinogenesis and molecular biology of the cancer cell, there has been a substantial increase in cancer research. This has resulted in the development of drugs that are targeted towards specific types of cancer, which may be very effective. Still, another approach has been to search for potential new anti-cancer drugs in natural resources such as land and sea plants, microorganisms, and animals [7]. MCF 7 (breast adenocarcinoma) is an established hormone responsive cell line which exhibits classical characteristics of differentiation in the presence of steroid hormones, cells are estrogen receptor (ER) positive and their proliferation can be inhibited by antiestrogen tamoxifen. These cells also possess mutant p53 gene and are also known for their cell adhesive properties. HCT 116 on the other hand is a human colorectal carcinoma derived from 116-year-old male. It is composed of moderately differentiated cells and is also known as it retains properties of glandular differentiation. These cell lines were selected mainly for the reason that they are most widely studied cell lines and any new drug or its derivative is often screened on these cell lines. With this background, the present work was undertaken to study cytotoxic activity of crude extracts of *Asplenium dalhousiae* and *Asplenium trichomanes* on MCF 7 and HCT 116 cell lines. The preliminary objective behind the

selection of these two *Asplenium* species was to determine the effect of cell treatment with the two *Asplenium* species on the cell viability and proliferation [8].

1.1 Research Objectives

The aim of this research was to evaluate the possible anti-cancerous effect of *A. dalhousiae* and *A. trichomanes*. This was carried out by testing the effect of the crude extracts on the growth of human breast and colon cancer cells in culture. It was hoped that this research would provide some scientific basis for the traditional use of these ferns in treating cancer, and thus may lead to the development of new cancer treatments. A simple method of assessing cytotoxicity that is now recognized as being able to effectively provide information on the growth of cancerous cells and compare it with non-cancerous cells is to use an MTT assay. This method measures the reduction of yellow MTT (3,4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide) by mitochondrial succinate dehydrogenase to form dark blue formazan which is largely specific to a viable cell and can be extracted with an organic solvent and then quantified by spectrophotometry. The assay was used to measure the final objective, growth inhibition of the cancer cells by the plant extracts, by comparing the optical density of treated and untreated cells.

2. METHODOLOGY:

2.1 Material Collection and Authentication

For this study, the ferns were collected from Malam Jabba, Swat Valley, which is a sky resort, 40 km away from Sadu Sharif, KP Pakistan. Dr. Zaheer-ud-din Khan, Distinguished Professor, Department of Botany, GCUL, identified both ferns using Flora of Pakistan and allotted the voucher numbers. The voucher specimens were submitted to Dr. Sultan Ahmed Herbarium with following voucher numbers:

GC.Herb.Bot.4003- *Asplenium trichomanes* L.
GC.Herb.Bot.4004- *Asplenium dalhousiae* Hook.

2.2 Preparation of Plant Extracts

Each fern plant was dried at room temperature in shady conditions and were separated to fronds, rhizome and sori. Each part was grounded to powder and was soaked in non-polar solvents (n-hexane and chloroform) for eight days in each, filtered and the residue shifted to the next solvent. After extraction in non-polar solvents, the residue was macerated in polar solvents (ethanol and distilled water) in same way. The powder to solvent ratio employed was (1:2). Extracts thus obtained were evaporated at 40°C using rotary evaporator. For evaluation of anti-cancerous activity, the stock solution of plant extract was prepared in 100 mg/ml concentration in DMSO.

2.3 Cytotoxicity Assay

The cytotoxicity and cell viability were tested in-vitro after [9] MCF-7 cell line was maintained in DMEM medium while HCT-116 in RPMI medium, both supplemented with 10% FBS and 1A antibiotics. The standard protocol to maintain these cell lines is 37°C with 5% CO₂ passage in humidified incubators. For assessment of anti-cancerous activity, 100 µL of cell line was seeded for 24 hours in 96-well microtiter plate. This incubation is necessary to adhere the cells to base of the plate. After this, 99 µL of culture medium and 1 µL of extract was poured using micropipettes. For control 1 µL of DMSO was used instead of plant extracts. This made the final volume 500 µL in each well. The plates were incubated for another 72 hours. After incubation 20 µL MTT was added to each well and incubated for another 4 hours. Afterwards, the culture medium was replaced by 100 µL DMSO to dissolve the crystals formed by MTT. Optical density was measured at 490 nm and percentage cell viability as well as percentage growth inhibition was calculated using formula:

$$\text{Growth inhibition (\%)} = 100 - 100 \times (T/C).$$

$$\text{Cell viability (\%)} = 100 - \text{GI}(\%)$$

Here C is the O.D. of cells treated with 0.5% DMSO while T is the O.D. of cells treated with plant extracts.

2.4 Statistical Analysis

The experiment was carried out in triplicates. Growth inhibition and cell viability percentages were presented as mean values with standard error and alphabetical subscripts to depict significant values. The standard error was calculated using formula in excel sheets while the mean values and alphabetical subscripts by Analysis of variance (ANOVA) with 5% Duncan's multiple range using co-stat (3.03 version) after [10].

RESULTS:

3.1 Cytotoxic Effects of Extracts against HCT 116

Table 1: Growth inhibition and cell viability of fronds, rhizome and sori extracts against HCT-116 Colorectal carcinoma cell line

Positive Control (Doxorubicin)		Growth Inhibition (%)		89.09±0.8%	
		Cell viability (%)		10.91±0.8%	
Negative control (DMSO)		Growth Inhibition (%)		0±0%	
		Cell viability (%)		100±0%	
		Antiproliferative activity of <i>Asplenium dalhousiae</i> Hook.			
		n-hex	Chl.	Ethanol	D. water
Fronds	Growth Inhibition (%)	54.63±0.23a	8.5±0.26l	15.16±0.42j	40.76±0.14c
	Cell viability (%)	45.36±0.23l	91.5±0.26a	84.8±0.42c	59.23±0.14j
Rhizom e	Growth Inhibition (%)	43.63±0.27b	10.73±0.12 k	18.66±0.14i	39.63±0.17d
	Cell viability (%)	56.36±0.27k	89.26±0.12 b	81.33±0.14d	60.36±0.17i
Sori	Growth Inhibition (%)	30.8±0.11f	22.8±0.11f	21.63±0.23h	37.76±0.14e
	Cell viability (%)	69.2±0.11g	77.2±0.11g	78.36±0.23e	62.23±0.14h
Growth inhibition LSD		0.307			
Cell Viability LSD		0.108			
		Antiproliferative activity of <i>Asplenium trichomanes</i> L.			
		n-hex	Chl.	Ethanol	D. water
Fronds	Growth Inhibition (%)	62.73±0.79d	29.8±0.11g	90.7±0.25a	8.63±0.17j
	Cell viability (%)	37.27±0.79i	70.2±0.11f	9.3±0.25l	91.36±0.17c
Rhizom e	Growth Inhibition (%)	44.7±0.80f	54.13±0.61 e	82.66±0.89b	8.7±0.25k
	Cell viability (%)	55.26±0.80g	45.86±0.61 h	17.33±0.39k	91.3±0.25b
Sori	Growth Inhibition (%)	21.8±0.11i	22.66±0.39 h	81.63±0.23c	4.76±0.14l
	Cell viability (%)	78.2±0.11d	77.33±0.39 e	18.36±0.23j	95.23±0.14a
Growth inhibition LSD		0.029			
Cell Viability LSD		0.0301			

*The alphabets depict significant differences (p<0.05) difference in mean values with Duncan's multiple range, ± specifies standard error. LSD is the least significant deviation.

Table 1 shows the anticancerous activity of non-polar and polar extracts of fronds, rhizome and sori extracts of *A. trichomanes* and *A. dalhousiae* against HCT- 116 cancer cell line.

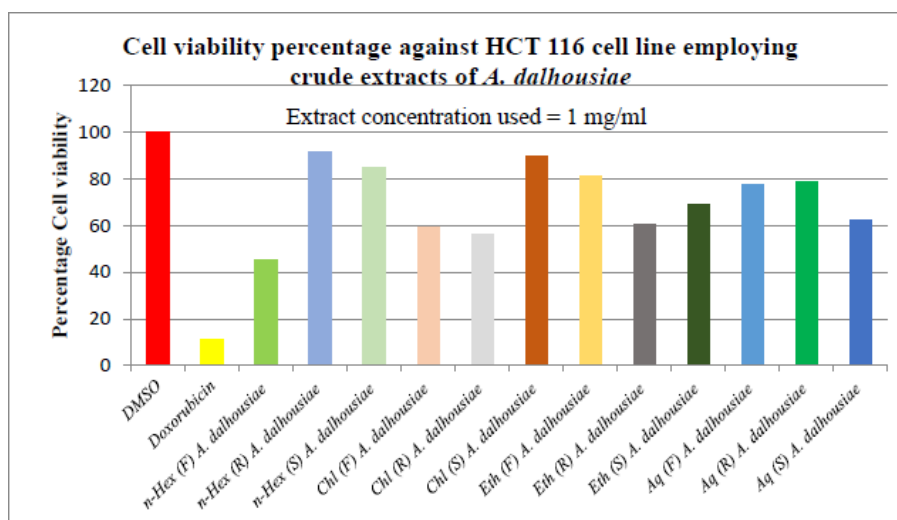


Fig. 1: Cell viability percentage against HCT 116 cell line employing crude extracts of *A. dalhousiae*

Extract of *A. dalhousiae* fronds in chloroform exhibited the lowest level of activity when tested against the HCT-116 cancer cell line, inhibiting just $8.5 \pm 0.26\%$ of the cells while leaving $91.5 \pm 0.26\%$ active. Likewise, the fern's n-hexane frond extract had the highest potency, exhibiting $54.63 \pm 0.23\%$ growth inhibition and $45.36 \pm 0.23\%$ cell viability (Fig. 1).

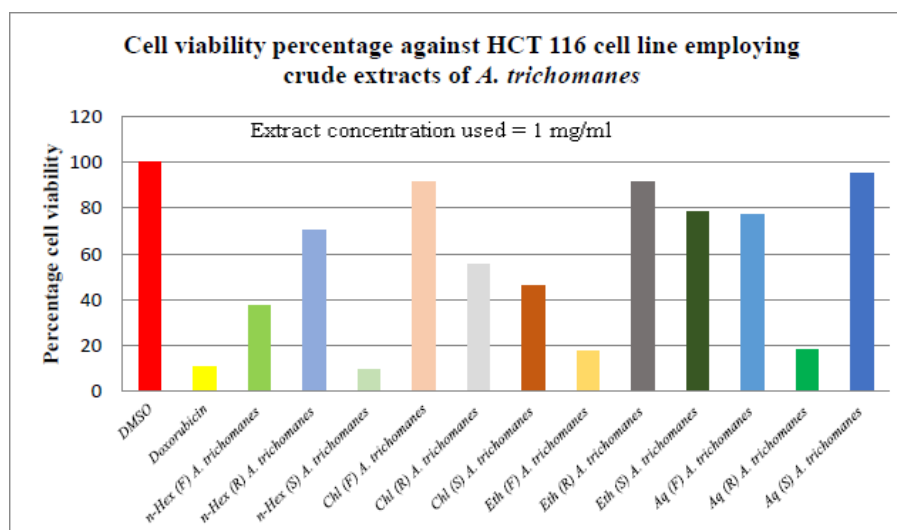


Fig. 2: Cell viability percentage against HCT 116 cell line employing crude extracts of *A. trichomanes*

With respect to the HCT-116, aqueous sori extract of *A. trichomanes* showed the least activity, inhibiting just $4.76 \pm 0.14\%$ of the cells while $95.23 \pm 0.14\%$ of the cells remained viable. Likewise, the fern's ethanol frond extract had the highest potency, exhibiting $90.7 \pm 0.25\%$ growth inhibition and $9.3 \pm 0.25\%$ cell viability (Fig. 2).

3.2 Cytotoxic Effects of Extracts against MCF 7

Table 2: Growth inhibition and cell viability of fronds, rhizome and sori extracts against MCF-7 Breast cancer cell line

Positive Control (Doxorubicin)		Growth Inhibition (%)		93.22±0.2%	
		Cell viability (%)		6.78±0.2%	
Negative control (DMSO)		Growth Inhibition (%)		0±0%	
		Cell viability (%)		100±0%	
		Antiproliferative activity of <i>Asplenium dalhousiae</i> Hook.			
		n-hex	Chl.	Ethanol	D. water
Fronds	Growth Inhibition (%)	41.63±0.23 b	14.66±0.39j	13.33±0.39h	47.9±0.20a
	Cell viability (%)	58.36±0.23j	85.33±0.29e	86.66±0.39d	52.1±0.20k
Rhizom e	Growth Inhibition (%)	19.66±0.39 e	7.23±0.14j	28.36±0.17c	9.36±0.17i
	Cell viability (%)	80.33±0.39 g	92.76±0.14b	71.63±0.17i	90.63±0.17c
Sori	Growth Inhibition (%)	41.73±0.12 b	6.73±0.12k	21.63±0.23d	17.73±0.12f
	Cell viability (%)	58.26±0.12j	93.26±0.12a	78.36±0.23h	82.26±0.12f
Growth inhibition LSD		0.190			
Cell Viability LSD		0.219			
		Antiproliferative activity of <i>Asplenium trichomanes</i> L.			
		n-hex	Chl.	Ethanol	D. water
Fronds	Growth Inhibition (%)	52.2±0.90f	61.7±0.25e	82.16±0.37a	3.7±0.25j
	Cell viability (%)	47.8±0.90g	38.3±0.25h	17.83±0.37l	96.3±0.25c
Rhizom e	Growth Inhibition (%)	42.03±0.52 g	67.03±0.52d	81.06±0.46c	2.73±0.21k
	Cell viability (%)	57.96±0.52f	32.96±0.52i	18.93±0.46j	97.26±0.21b
Sori	Growth Inhibition (%)	11.26±0.12i	28.06±0.46h	81.7±0.25b	0.26±0.12l
	Cell viability (%)	88.73±0.12 d	71.93±0.46e	18.3±0.25k	99.73±0.12a
Growth inhibition LSD		0.249			
Cell Viability LSD		0.285			

*The alphabets depict significant differences ($p < 0.05$) difference in mean values with Duncan's multiple range, $\pm$ specifies standard error. LSD is the least significant deviation.

Table 2 shows the anticancerous activity of non-polar and polar extracts of fronds, rhizome and sori extracts of *A. trichomanes* and *A. dalhousiae* against MCF-7 cancer cell line.

A. dalhousiae chloroform sori extract inhibited just 6.73±0.12k% of the MCF-7 cancer cell line, leaving 93.26±0.12a% of the cells alive. This extract was the least active against this cell line.

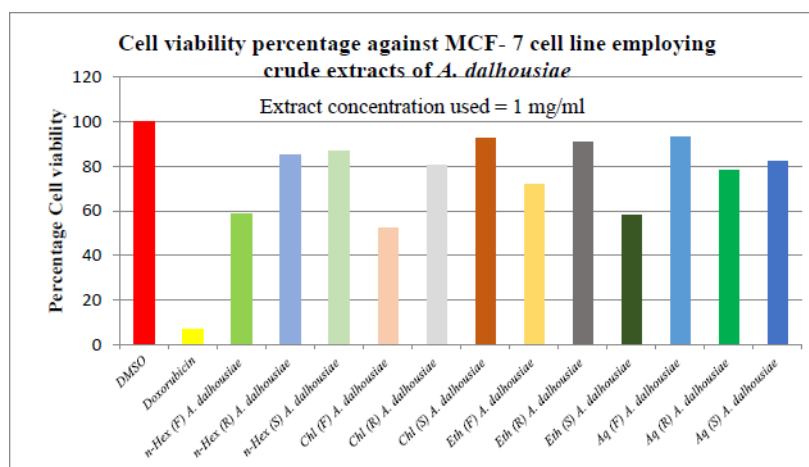


Fig. 3: Cell viability percentage against MCF- 7 cell line employing crude extracts of *A. dalhousiae*

Likewise, the fern's aqueous frond extract had the highest potency, exhibiting $47.9 \pm 0.20\%$ growth inhibition and $52.1 \pm 0.20\%$ cell viability (Fig. 3).

The MCF-7 Breast Cancer Cell Line showed the least response to *A. trichomanes* aqueous sori extract, which inhibited just $0.26 \pm 0.12\%$ of the cells while $99.73 \pm 0.12\%$ of the cells remained viable.

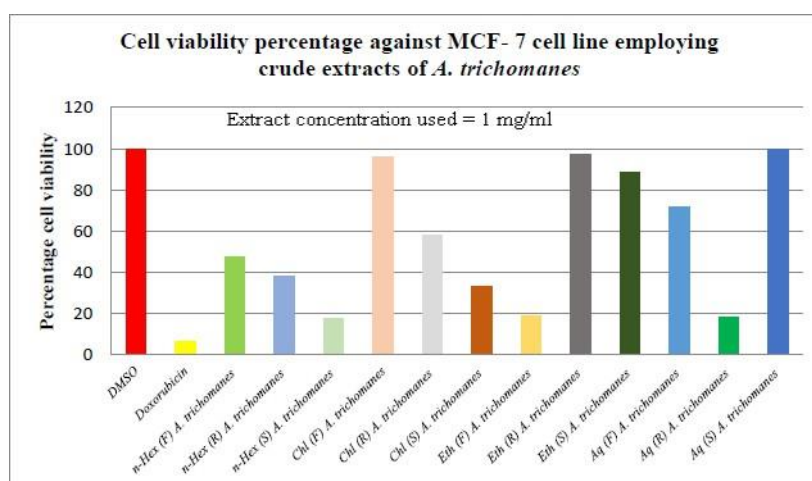


Fig. 4: Cell viability percentage against MCF- 7 cell line employing crude extracts of *A. trichomanes*

Parallel to this, the fern's ethanol frond extract had the highest potency, exhibiting $82.16 \pm 0.37\%$ growth suppression and $17.83 \pm 0.37\%$ cell viability (Fig. 4).

4. DISCUSSION

HCT-116 cells are derived from the p53-null HCT 8 cell line. These cells have a stable karyotype and have also been utilized in multiple in vitro studies. The HCT-116 cell line is heterogeneous and possesses normal as well as chromosome 3-derived segments in the q-arm of chromosome 2, to the q-arm of chromosome 10. It is a medium-sized cell line with centrally located nuclei and minimal cytoplasm. They are a highly invasive, adherent cell line which forms colonies in Delfia while remaining as individual cells in culture. These cells contain wild type p53 and express the p53 mRNA and protein constitutively [11]. They show a 20-hour duplication time and are used for transfection studies. This cell line has been widely used in cancer biology studies due to its natural genotypic

heterogeneity. The skeletal infrastructure of HCT-116 cell lines will activate components known to initiate a plethora of oncogenic pathways when these cells are injected into a host.

Both MCF-7 and HCT-116 cell lines are human cancer cells, but originated from different tissues. MCF-7 cells are derived from breast carcinoma tissue of a 69-year-old Caucasian woman. The cells have been found to have estrogen receptors and are also the first cultured cell lines said to be estrogen receptor-positive. They have an epithelial morphology; their microscopic examination indicated cuboidal and round shapes connected by desmosomes. MCF-7 cells are sensitive to estrogen and the steroidal estrogen estradiol, and respond with high proliferation rates and express hormone-inducible genes [12]. With tamoxifen treatment and estrogen deprivation, these cell lines become apoptotic.

In modern times, natural products have reappeared as the chief source of medicinal properties. Furthermore, plant-derived compounds or extracts, through their pharmacological or chemotherapeutic activity, are of great interest. Herbal therapy can hence be used in the future as an additional option, particularly in cases of unusual, resistant, or untreatable cancer. Ferns are used not only for food, but also for health and medicinal requirements. Modern analytical techniques can be used to determine the active principle and extract, which could be used to treat various diseases distorted by tracts and substances for a particular ailment. Some of this information is recorded in folklore. Our current work is an attempt to demonstrate that bioactive components that act as a chemotherapeutic agents are present in *Asplenium trichomanes* & *Asplenium dalhousiae*, which have unknown structural formulas and which can be extracted with different solvents including ethanol. These extracts have cytotoxic activity, as confirmed by lowering cell growth and cell viability. The crude extracts were tested against MCF-7 and HCT-116 cell lines maintained in RPMI and DMEM medium. The extracts of both ferns demonstrated cytotoxic activity. The chloroform and ethanol extracts exhibited noticeable growth inhibitory activities on both MCF-7 and HCT-116 cells. Previous studies on anticancerous activity of other *Asplenium* genus members on MDA-MB-231 cell line showed reduction in proliferation [13][14][15]. These results may help us make them a potential source of novel anticancer drugs. Our study convincingly showed that both fern's chloroform extracts can be potentially used for the development of novel anticancer drugs. The major concern of anticancer drugs nowadays is the enormous side effects and alterations of drug responses over time. Natural compounds derived from various kinds of plants have long been utilized in traditional plant-based medicine for the treatment of malignancies. These compounds have shown to have beneficial effects such as cellular differentiation, antiangiogenic and antimetastatic properties.

5. CONCLUSION

Herbal medicine may have an increased effect as a complementary treatment rather than a main therapy for alleviating cancer therapy-related side effects. Additionally, the combination of clinically established medications with phytopharmacological components may enhance long-term effects when used as a complementary treatment for cancer. Plants with anticancer compounds have made significant contributions to the field of cancer research and have been scientifically accredited for several centuries. Numerous studies, clinical evidence, compelling hypotheses, officially accepted therapeutics, and regulatory opportunities support the use of these natural compounds. Our study concluded that both *A. trichomanes* and *A. dalhousiae* extracts were found potential candidates for anticancer activities.

6. DECLARATION OF FUNDING

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