

Nutraceutical Composition and Anti-Cancerous Potential of an Unexplored Herb *Asplenium ceterach* from Kashmir Region

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ABSTRACT

Despite significant breakthroughs in understanding, prevention, and treatment of prostate cancer, it still represents the fourth leading cancer in the general population worldwide. The high rate of mortality among prostate cancer patients is clear indication of insufficient efficiency of currently available prostate cancer therapies. Thus, there is a constant demand for new therapies or drugs to treat and prevent this life-threatening disease. Identifying new chemotherapeutic agents with fewer side effects is a major concern for scientists today. In this direction, medicinal herbs are nowadays looked to have great potential to act as chemopreventive agents. One such underutilized potential medicinal herb *Asplenium ceterach* is highly unexplored in Kashmir region. Therefore, the present investigation was undertaken to investigate the nutraceutical composition, antioxidant activity and preliminary anti-cancerous potential of this medicinal herb. The current study demonstrated aqueous extract of *Asplenium ceterach* (ACE) to be rich source of nutraceuticals like phenols ($465 \pm 1.49\text{mg per } 100\text{g}$) and flavonoids ($66.34 \pm 0.351\text{mg/ } 100\text{g}$) and possess strong antioxidant activity. This preliminary study demonstrated remarkable anti-cancerous activity of AC extract against two prostate cancer cell lines viz., PC3 and LNCaP. This research study provides a scientific basis for exploitation of *Asplenium ceterach* from Kashmir valley for its use for anti-cancer chemopreventive drug development or as an adjuvant to existing prostate cancer therapies.

Keywords: *Asplenium ceterach*, Nutraceuticals, Anti-oxidant, Prostate cancer, Anti-cancer.

INTRODUCTION

In the present era, cancer is a global health issue with severe detrimental effects on human health. According to the latest report of International Agency for Research on Cancer

(IARC) of World Health Organization (WHO), the global cancer burden is estimated to have risen to ~18.1 million new cases (IARC, 2018).

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Among the major cancer types in men, a recent report of American cancer society (2018) has listed prostate cancer as one of the most common cancers, with approximately 1,64, 690 new cases (9.5%) in the United States in 2018, and thus represents it as the fourth leading cancer in the general population. As far as India is concerned, a recent data published in Indian express (2018) states that prostate cancer is the second most common cancer in Indian males as per ICMR and various state cancer registries. The prostate cancer patients suffer greater mortality rates, with an estimated death rate of 4.8% (29, 430 cases) in 2018 and is thus, a clear indication of insufficient efficiency of the currently available prostate cancer therapies (National Cancer Institute, 2018). The treatments including medication, surgery and radiation, used against prostate cancer often come up with unwanted side effects such as drug resistance (especially in metastatic prostate cancers), with their ever-increasing costs and the worse survival rates in both developing as well as in the developed nations (Holm et al., 2017). Therefore, the need of hour is to continue the search for some much safer and more effective alternate chemo-preventive agents or therapies against this disease. In this regard, natural plants or medicinal herbs have a great potential in curbing such oxidative stress related disorders. Recent scientific data clearly indicates that medicinal herbs, in addition of being a rich source of nutrients, represents a reserve of numerous secondary metabolites as nutraceuticals for the development of novel and safer drugs with minimal side effects (Saeed et al., 2018). Nowadays, use of these natural plant products (such as crude plant extracts) or combination of different plant chemicals as a new source of potent and novel anti-cancerous prototypes has been a point of focus (Altemimi et al., 2017). In this context, some underutilized herbs have been recently reported to possess enormous anti-cancerous potential and thus, necessitates their proper exploitation (Kour et al., 2018). One of such category is represented by various underutilized species of *Asplenium* genus.

Among them, *Asplenium ceterach* has recently gained a great deal of attention from researchers worldwide, due to its rich nutritional profile and phytochemical composition (Zivkovic et al., 2017). Therefore, the aim of current study was to investigate the nutraceutical composition, antioxidant activity and anti-cancerous potential of *Asplenium ceterach* grown in Kashmir region.

MATERIALS AND METHODS

Collection of Plant Material

The dried whole plant samples of *Asplenium Ceterach* were collected from Regional cum Facilitation Centre (RCFC), Faculty of Agriculture, SKUAST-K, Kashmir, India.

Preparation of Lyophilized AC extract

The collected plant samples of *Asplenium Ceterach* were first separated from unwanted dirt and other foreign material followed by crushing it to a fine powder in a grinding machine. The powdered sample (500gms) was then soaked over night in 1000 ml of distilled water at room temperature with occasional shaking or stirring. Finally, it was homogenized using a tissue homogenizer (WiseTis HG-15A Homogenizer, Switzerland) and filtered through muslin or cheese cloth. The procedure was repeated five times; each in 48 hours to ensure complete extraction and the whole extract was then pooled, filtered (Whatman filter paper No.1) and lastly concentrated under vacuum at 40°C using rotatory evaporator (Buchi). The concentrated aqueous extract obtained was subjected to lyophilization using a freeze drier (Scope enterprises, USA). After lyophilization of the aqueous extract, 420 mg of lyophilized powder of *Asplenium Ceterach* extract (ACE) was obtained and stored at -20°C in sealed bottle till further analysis.

Phytochemical analysis of Asplenium ceterach extract (ACE)

Preparation of plant extract for nutraceutical

analysis: For nutraceutical analysis, 10mg of lyophilized powder of *Asplenium Ceterach* was dissolved in 10 ml of double distilled water and used directly for further analysis.

Estimation of total Phenol The total phenolic content of lyophilized extract of *Asplenium Ceterach* was determined by modified method of Malick and Singh (1980).

Estimation of total flavonoids Total flavonoid content of lyophilized extract of *Asplenium Ceterach* was determined according to modified method of Lallianrawna et al. (2013).

Estimation of total anti-oxidant activity The total antioxidant potential of lyophilized extract of *Asplenium Ceterach* was determined by using the ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996) as a measure of its antioxidant power.

Anticancerous Activity of *Asplenium ceterach* extract (ACE)

Preparation of plant extract for Anticancerous Activity For evaluation of anticancerous potential, 10 mg of lyophilized powder of *Asplenium Ceterach* was dissolved in molecular biology-grade DMSO and diluted in RPMI 1640, in order to prepare four different concentrations of AC extract (1.5mg/ml, 3mg/ml, 6mg/ml and 12mg/ml). The AC extract was then stored at 4°C till further analysis.

Cell lines and culturing Human prostate cancer (PCa) cell lines (PC3 and LNCaP) used in this study were procured by Transcriptomis laboratory, Division of Plant Biotechnology, SKUAST-K from National Animal Cell Repository at National Center for Cell Science, Pune. Prostate epithelial cells served as representative of normal cells in this study. All the cells were separately cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (Invitrogen), 2mM L-glutamine, 50 IU/ml penicillin, 50µg/ml streptomycin, and maintained at 37°C in humidified 5% CO₂ atmosphere throughout the assay.

Clonogenic assay or Colony formation assay The colony formation assay using logarithmically growing cells was performed according to the method of Koppikar et al. (2010). Briefly, the cells were plated in the RPMI medium at a seeding density of 1×10^3 cells in each well of the microtiter plates (E-plates 6), followed by incubation for 24 h at

37°C in humidified 5% CO₂. After 24 hours, the medium was removed and the cells were treated with 1ml of different concentrations of the ACE (1.5mg /ml, 3mg/ml, 6mg/ml, and 12g/ml). Control cultures received only vehicle (0.1 % DMSO) in place of extract and doxorubicin treated cells served as positive control. After treatment, the cells were incubated with fresh media for 7 days, fixed with methanol: acetic acid (3:1) for 5 min and stained with 0.5% crystal violet in methanol. The cell colonies with more than 50 cells / well were selected and counted under a light microscope (Olympics, USA). Colonies of at least 10 different random fields were counted for each well and averaged. The experiment was performed in triplicate and results expressed as mean ± SD.

In Vitro Wound-Healing Assay (Scratch test)

The migration rates of PCa cell lines (PC3 and LNCaP) was assessed by scratch wound healing assay method. The assay was performed as described previously by Chen et al. (2011). Briefly, the cells were plated in RPMI medium at a seeding density of 5.5×10^5 cells in each well of the microtiter plates (E-plates 24) followed by incubation at 37°C in humidified 5% CO₂ till the cells reached ~100% confluence. Further, the cells were serum starved for 24 h and after that, the monolayer was scratched with a sterile pipette tip (200 µL). The cells were washed with PBS (pH 7.4) to remove floated or detached cells and photographed (time 0 h). Cells were successively treated with medium containing low serum (1.0%) and different concentrations of AC extract (1.5µg /ml, 3µg/ml, 6µg/ml, and 12µg/ml) and incubated for 24 h, along with the cells treated separately with DMSO only. Wounded areas were photographed at a 400µm magnification using EVOS FL Auto Cell Imaging system (Thermo Fisher Scientific, Waltham, MA, USA) after 48 hours of treatment. The experiments were performed in duplicate.

Statistical analysis

The statistical analysis of data generated in the current study was carried out by means of one way analysis of variance (ANOVA) and the

comprehensive statistical package SPSS (Version 20) for windows was used.

RESULTS AND DISCUSSION

Cancer, a life threatening disorder severely affecting the mankind, is one of the most serious global issues in today's world. Among the major cancer types in men, a recent report of American cancer society (2018) has listed prostate cancer as one of the most common cancers. Despite extensive research on the discovery of anti-cancerous drugs against this cancer, the incidence and prevalence of prostate cancer remains high and in many cases, drug resistance leads to lack of appropriate response. In the present era, underutilized medicinal plants are being viewed as an alternate and safer source of medicinal compounds, and if properly exploited can lead to the development of novel therapeutics or drugs (Mushtaq et al., 2018). Recently some *Asplenium* species have been found to possess cytotoxic effects against several cancer cell lines (Jarial et al., 2016). In this context, an underutilized herb namely *Asplenium ceterach* traditionally been used as a diuretic, anti-oxidant, anti-microbial and astringent agent in folk medicine possess great potential (Wani et al., 2016). However, to the best of our knowledge, not even a single report have been published till date from Kashmir region evidencing such properties, especially anticancerous potential of *Asplenium ceterach*. Therefore, in the current study, the nutraceutical composition, antioxidant potential and anti-cancerous properties of *Asplenium ceterach* grown in Kashmir valley were studied.

Epidemiological studies have shown that fruits and vegetables exhibit numerous biological properties including anticancer, antiviral, antioxidant and anti-inflammatory activities, due to their rich nutraceutical composition, especially polyphenolic compounds (Kibe et al., 2017). Therefore, in the current investigation, the aqueous extract of *Asplenium ceterach* was also evaluated for its total phenol content and demonstrated 465 ± 1.49 mg per 100g Gallic acid equivalent of

total phenols on dry weight basis (Table 1). These results are in strong agreement with Ondo et al. (2013) who reported 341.16 ± 0.06 mg/100g of gallic acid equivalents of total phenols in hydro alcoholic extract of leaf samples in *Asplenium africanum*. Though, similar observations were also reported by Ullah et al. (2018) in *Asplenium dalhousiae* collected from different areas of district Dir Lower, Pakistan but with lower levels (32 to 168 mg/ 100g).

These Polyphenols actually represents a diverse group of compounds including simple phenols, flavonoids (flavonols, flavones, flavonones, isoflavones and anthocyanins), hydroxybenzoic acid, hydroxycinnamic acid, chalcones, aurones, hydroxycoumarins and hydroxystilbenes (Liu et al., 2016). Among these polyphenols, the flavonoids are widely distributed in plants and recognized for their anti-oxidant, anti-hyperglycemic and anti-proliferative effects (Wang et al., 2017). As shown in Table 1, the aqueous extract of *Asplenium ceterach* demonstrated 66.34 ± 0.351 mg/100g of total flavonoids on dry weight basis. These results are more than two times higher than the findings of Berk et al (2013) who reported 29.69 ± 0.11 μ g/mg quercetin equivalents of total flavonoids present in the aqueous extract of *Asplenium ceterach*. The current results are in good agreement with the observations of Zivkovi et al. (2017).

Accumulating scientific evidence suggests that the over-production of reactive oxygen species (ROS) may be the root cause of several chronic diseases such as cancer, cardiovascular diseases, neurodegeneration and aging in humans (Huang, 2018). The problem of this overproduction of ROS can be circumvented through the dietary intake of natural antioxidants (Liu et al., 2018). Phenolics and flavonoids from plants are nowadays commonly known as the largest group of nutraceutical molecules with strong antioxidant properties. Therefore, in the current study, the nutraceutical rich aqueous extract of *Asplenium ceterach* was evaluated for its total antioxidant activity that

ranged from 29.36 ± 0.32 mM Fe²⁺/100g FRAP on dry weight basis (Table 1). These results are much higher than the findings of Kazazic et al. (2016) who reported total antioxidant

activity of aqueous extracts of *Asplenium ceterach* to be 12.26 ± 0.15 mM Fe²⁺/ 100 g DW and can thus serve as potential antioxidant herb.

Table 1: Total phenolics, Total flavonoids content and Total antioxidant activity of Aqueous extract of *Asplenium ceterach*

Total phenolic content*	Total flavonoid content**	Total antioxidant activity (FRAP) ***
465±1.49	66.34 ± 0.351	29.36±0.32

*mg gallic acid equivalent (GAE)/100g DW, **mg quercetin equivalent/100g DW, ***mM Fe²⁺/100g DW. Values are mean± SD of three biological replicates.

Continual unregulated proliferation of cancerous cells is the fundamental abnormality in the development and progression of cancer, and this often leads to tumor clonality (Parsons, 2018). Exploitation of plants rich in bioactive constituents and their inclusion in our daily diet have been found to be quite beneficial for cancer prevention and treatment, as they have been described to block or inhibit the proliferative integrity of individual cancer cells under cell cultured conditions (Al-Hasawi et al., 2018). In the current study, the anti-proliferative activity was determined by clonogenic cell survival assay by observing the ability of *A. ceterach* extract to arrest the indefinite proliferation of cells by reducing their reproductive viability. As shown in Table

2, the survival fractions of two types of highly metastatic prostate cancer cell lines (PC3 and LNCaP) after exposure to different concentrations of aqueous extract of *Asplenium ceterach* (ACE) demonstrated decreased clonogenic survival. The normal prostate epithelial cells, on treatment with extract of *Asplenium ceterach* showed less magnitude effects (Data not shown). Remarkably, the two cancer cell lines exhibited a dose dependent inhibition in their proliferative/colony formation capacity at different ACE concentrations. These results, thus provided a scientific basis for the antiproliferative and anticancerous effects of the aqueous extract of *Asplenium ceterach*.

Table 2: Number of colonies formed after 7 days in each cell line pretreated with four different concentrations of *Asplenium ceterach* (ACE)

S. no	Treatment (µg/ml)	Colonies	
		PC-3 cell line	LNCaP cell line
1	0 (DMSO)	121	114
2	1.5µg/ml	86	72
3	3µg/ml	49	46
4	6µg/ml	31	28
5	12µg/ml	19	13

The proliferation of cancer cells from primary tumors and their subsequent seeding of new tumor colonies in distant tissues involve a multi-step process known as the invasion metastasis cascade. Anti-metastasis therapy is a new approach to cancer treatment, requiring agents to retard the invasion of cancer cells, Copyright © March-April, 2020; IJPAB

which have little or no cytotoxic activity (*Lirdprapamongkol* et al., 2008). The current study was carried further ahead with wound healing assay to check the anti invasive ability of aqueous extract of *Asplenium ceterach* against two cancer cell lines (PC3 and LNCaP). Our results demonstrated that

treatment with different concentrations of *Asplenium ceterach* extract for 48 h effectively inhibited the migration of all the two cell lines (PC3 and LNCaP,) in a dose- and time-dependent manner compared to the untreated control cells (Figure 1). The control cells (without treatment) were observed to attain almost 100% confluence and wound was healed almost completely after 48 hours.

However, treatment with *Asplenium ceterach* extract resulted in inhibition of cell migration. Treatment with 2µg/ml concentration of extract was found to have insignificant effects. However, 12µg/ml AC extract treatment significantly decreased the migration rate of PCa cells lines, thereby affecting their migration capability and the wound healing ability.

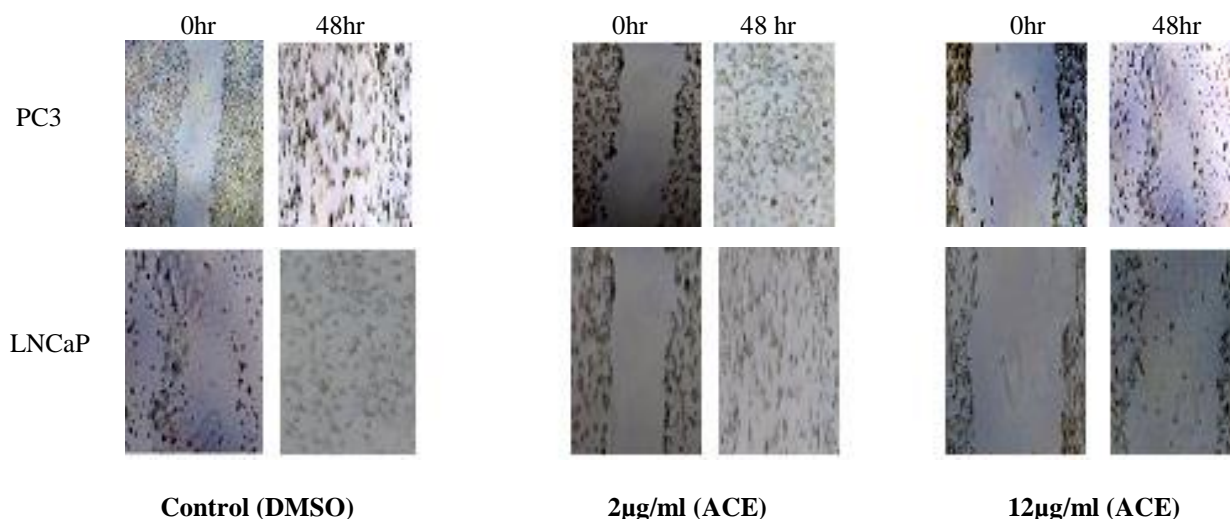


Figure 1: Photomicrographs indicating effect of ACE on wound closure of PC3 and LNCaP cancer cell lines after 48hrs.

The present investigation clearly suggests that ACE treatments significantly inhibited motility of PCa cell lines in statistically significant ($p \leq 0.05$) manner (Figure 1). Similar kind of observations were also recorded by Antony Samy et al (2014) while studying the cytotoxic effects of crude acetone extracts of *Asplenium aethiopicum* that caused 50% and 90% mortality of brine shrimp nauplii at the respective concentrations of 192.8 and 434.3 ppm.

CONCLUSION

In conclusion, our study demonstrated that *Asplenium Cetrech* is a rich source of nutraceuticals, exhibits strong antioxidant potential and possess anti-cancerous activity under *in vitro* conditions against two prostate cancer cell lines (viz., PC3, and LNCaP cell lines) in a dose dependent manner. However, the current study warrants a detailed investigation to elucidate the exact mechanism of action of *Asplenium Cetrech* against cancer,

so as to exploit this herb as a nutraceutical rich source of anti-cancer drug or as an adjuvant to existing prostate cancer therapies.

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