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ORIGINAL ARTICLE

Phytochemical Standardization of Panchavalkala: An Ayurvedic Formulation and Evaluation of its Anticancer Activity in Cervical Cancer Cell Lines

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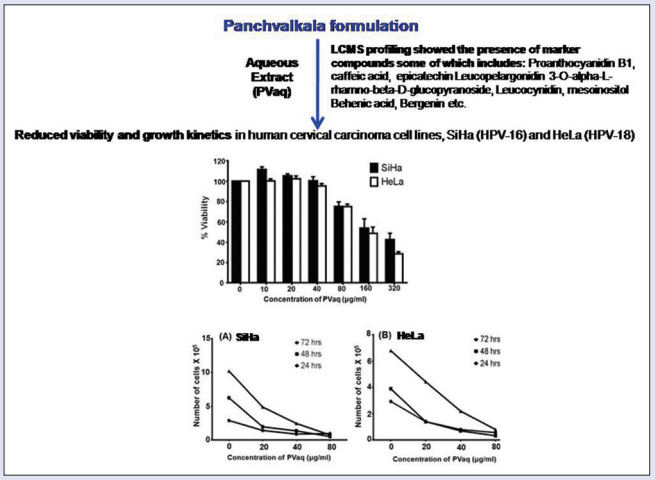
ABSTRACT

Background: Cervical cancer is the most common malignant disease affecting women worldwide. The currently available therapies for cancer, even though effective, affect the patient's health severely due to the associated side effects. Thus, nowadays, complementary/alternative medicines are being extensively researched upon for their use as an adjunct therapy. Panchavalkala, an Ayurvedic formulation, is traditionally being used as a douche in leukorrhea and other gynecological diseases. **Objective:** The objective of the study was to phytochemically standardize aqueous extract of Panchavalkala (PVaq) and evaluate its anticancer activity against human cervical cancer cell lines. **Materials and Methods:** The phytochemical characterization of PVaq was done by liquid chromatography–mass spectrometry (LCMS) technique. The effect of PVaq on the viability of SiHa and HeLa cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide dye assay. The effect of the extract on growth kinetics was evaluated by trypan blue dye exclusion method and soft agar assay. **Results:** LCMS analysis showed presence of 77 compounds, of which 15 major compounds included proanthocyanidin B1, chlorogenic acid, caffeic acid, epicatechin, leucopelargonidin 3-O-alpha-L-rhamno-beta-D-glucopyranoside, leucocyanidin, naringenin-7-o-glucoside, mesoinositol, catechin, vogelin E, mesoinositol, behenic acid, bergenin, acacetin, and gallic acid. PVaq significantly ($P < 0.001$) reduced the viability of SiHa and HeLa cells with an IC_{50} of 125.8 and 96.0 µg/ml, respectively. It also reduced the growth of cervical cancer cells in a dose- and time-dependent manner. **Conclusion:** This preliminary data suggests that PVaq exhibits potential anticancer activity and warrants further studies for detailed elucidation of its mechanism of action.

Key words: Aqueous extract of Panchavalkala, cell viability, cervical cancer, growth kinetics, liquid chromatography–mass spectrometry

SUMMARY

- In the present study, we have done phytochemical standardization of aqueous extract of Panchavalkala (PVaq) and evaluated its anticancer activity against cervical cancer cell lines. PVaq showed presence of the phytocompounds having reported antioxidant, anti-inflammatory, and anticancer activities
- PVaq decreased the viability of human papillomavirus-positive cervical cancer cells in a dose-dependent manner
- It altered the growth kinetics of the cancer cells in a dose- and time-dependent manner.



Abbreviations used: PVaq: Aqueous extract of Panchavalkala; LCMS: Liquid chromatography–mass spectrometry; HPV: Human papillomavirus; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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INTRODUCTION

Cervical cancer is the second most leading cause of cancer death among Indian women.[1] Every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease.[1]The persistent infection with human papillomavirus (HPV), notably type 16 and 18 has been found to be the primary cause for cervical cancer.[2] The available conventional therapies which include surgery/chemotherapy/radiotherapy have greatly reduced the mortality; however, they are associated with serious adverse events.[3,4] Nowadays, cancer research has shifted its focus towards identification of herbal based drugs that have no or minimum

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side effects; exhibit anticancer activity; and could be used as adjunct to conventional therapies.

Panchavalkala is an Ayurvedic formulation comprising equal proportions of bark materials of *Ficus benghalensis*, *Ficus virens*, *Ficus religiosa*, *Ficus glomerata,* and *Thespesia populnea*. Traditionally, it has been used for the treatment of female infertility and endometriosis-related problems.[5-10] The decoction has been extensively used as a douche in leukorrhea and other vaginal diseases.[6-10] It has also been reported that regular douching of the genital tract with the decoction of Panchavalkala helps in reduction of symptoms related to vaginitis.[6-10] In addition, the individual components of the formulation have well reported anticancer activity.[11-13] In the present study, we have for the first time performed liquid chromatography–mass spectrometry (LCMS) profiling of aqueous extract of Panchavalkala (PVaq) and evaluated its anticancer activity against HPV16+ (SiHa) and HPV18+ (HeLa) cervical cancer cell lines. LCMS profiling revealed the presence of compounds with reported anti-inflammatory, antioxidant, and anticancer properties. Treatment of SiHa and HeLa cells with PVaq significantly reduced their viability and growth kinetics, thereby signifying the anticancer potential of the formulation.

MATERIALS AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM) powder, penicillin and streptomycin were purchased from Invitrogen/Gibco (Grand Island, NY, USA). Agarose was purchased from Gibco (DNA grade, BRL, CA, USA). Fetal bovine serum (FBS) and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tissue culture plasticware was purchased from BD Biosciences (San Diego, CA, USA). The human cervical carcinoma cell lines, SiHa (HPV-16) and HeLa (HPV-18), were obtained from National Center for Cell Science, Pune, Maharashtra, India. The cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and streptomycin). The cells were maintained in a humidified 5% CO_2 incubator at 37°C.

Plant material and extraction

Barks of *F. religiosa L.* (Auth. 17-256), *F. virens* (Auth. 17-252), *F. glomerata* (Auth. 17-251), *F. benghalensis* (Auth. 17-250), and *T. populnea* (Auth. 17-253) were collected from Pune, Maharashtra, India. Botanical identification of plant material was carried out at Agharkar Research Institute, Pune, Maharashtra, India. The barks of all the five plants were chopped into small pieces, shade dried at ambient temperature, and stored in airtight container. It was ground into coarse powder in a grinder whenever required. The powders from each bark were taken in the 1:1 ratio for extract preparation. The extract obtained was centrifuged at 13,000 rpm for 15 min, and the supernatant was filtered through Swiney filter (pore size, 0.45 µm) and stored at −80°C until further use. Aqueous extract of the mixture of powders was prepared as per the standard procedure for preparation of kwath, i.e., aqueous extract given in Ayurvedic Formulary of India (deduced from Charaka Samhita, Sutrasthan Adhyay 4;8).

Liquid Chromatography–mass spectroscopy

Non-targeted and targeted HPLC-MS QTOF analysis was performed as described earlier.[14] Briefly, 10 µL of sample was injected onto an Agilent 1290 HPLC system having Zorbax Eclipse Plus C18 column (2.1 mm × 100 mm, 1.8 µm particle sizes). The mobile phases consisted of (A) water and (B) acetonitrile (LCMS grade, J. T. Baker) with flow rate of 0.3 mL/min and 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) formic acid for MS analysis in positive mode and with 5 mm ammonium acetate for analysis in negative mode. The chromatographic conditions utilized for the study consisted of the first 5 min run isocratically at 5% B; a gradient of B from 95% to 5% was applied from 5 min to 30 min, followed by 3 min isocratically at 100%. MS analysis was performed on an Agilent 6530 Quadrupole time-of-flight spectrometer fitted with an electrospray ionization source in both positive and negative mode. Data were analyzed using Mass Hunter Qualitative Analysis Software Package (Agilent Technologies) and online database Metlin. Blanks using each of the solvent extraction systems were analyzed using "Find by Molecular Feature" algorithm in the software package to generate a compound list of molecules with significant abundances >10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. The data were analyzed using "Find by Molecular Feature" function to generate a list of compounds with empirical formula in the extracts. Compound lists were then screened against online mass databases; METLIN Metabolomics Database and MassBank Database.[15]

Cell viability assay

SiHa and HeLa cells were seeded at a density of 1×10^5 cells/ml in 96-well plates. The cells were treated with different concentrations (0, 10, 20, 40, 80, 160, 320, and 640 µg/ml) of PVaq in each well in triplicates for 24 h. Cell viability was determined by MTT assay as described previously.[11,16]

Cell growth analysis

SiHa and HeLa cells were seeded at a density of 1×10^5 cells/ml in 24-well plates in triplicates. Next day, the cells were treated with different concentrations of PVaq (0–320 µg/ml) for 24, 48, and 72 h. The cells were harvested and counted for viability with trypan blue dye using a hemocytometer.[11,16]

Soft agar assay

SiHa and HeLa cells (5×10^3 cells/ml) treated with different concentrations of PVaq (0–320 µg/ml) were mixed at 40°C with 0.35% agarose in culture medium and gelled at room temperature for 20 min over a previously gelled layer of 0.5% agarose in culture medium in 6-well plates. After incubation for 2 weeks, the colonies were counted in ten different fields using an Axiovert 200M microscope (Carl Zeiss, Germany), and the average value was calculated.[11,17]

Statistical analysis

All the results were obtained from three independent experiments, each performed in triplicates and the values have been presented as mean ± standard deviation. Differences among means were tested for statistical significance using one-way analysis of variance. The analyses were carried out using GraphPad Prism 5 software (San Diego, CA, USA). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered to be statistically significant.

RESULTS

Liquid Chromatography–mass spectroscopy analysis

LCMS analysis of PVaq showed the presence of a range of phytochemicals, which included high levels of phenolics and flavonoids and moderate levels of tannins. A total of 77 compounds were identified [Supplementary Table 1]. The peaks of the compounds were obtained at different retention times. The highest peak was at the retention time of 15.54, followed by retention times of 15.11, 10.61, 8.74, 6.95, 5.14, 4.72, 2.97, 2.83, 0.98, 0.64, and 0.45, corresponding to the compounds behenic acid

Table 1: Major compounds present in panchavalkala identified by liquid chromatography-mass spectroscopy

Name of identified compound	Empirical formula	Observed RT	*M/Z* value	Observed mass	Abundance
Behenic acid	C22 H44 O2	15.54	340.33413	340.3335	-
Vogelin E	C20 H18 O6	15.11	354.11033	354.1079	-
Acacetin	C16H12O²	10.61	284.06847	284.0678	69350.3
Leucopelargonidin 3-O-alpha-L-rhamno-beta-D-glucopyranoside	C27H34O15	8.74	598.18977	598.1871	1073
Naringenin-7-O-Glucoside	C21 H22 O10	6.95	434.12129	434.1209	22504.89
Epicatechin	C15 H14 O6	5.14	290.07903	290.0787	1517.73
Proanthocyanidin B1	C30H26O12	4.72	578.14242	578.1401	1228.51
Chlorogenic acid	C16H18O9	2.97	354.09508	354.0941	2203.07
Bergenin	C14H16O9	2.83	328.07943	328.0803	5188
Leucocynidin	C15 H14 O7	0.98	306.07395	306.0761	-
Gallic acid	C7H6O²	0.64	170.02152	170.0214	1107.61
Mesoinositol	C6H12O6	0.45	180.06338	180.0632	4257.39
Caffeic acid	C9H8O4	2.36	180.04225	180.0423	-
Catechin	C15 H14 O6	5.14	290.07903	290.0787	1517.73

RT: Retention time

(m/z 340.33413), Vogelin E (m/z 354.11033), acacetin (m/z 284.06847), leucopelargonidin 3-O-alpha-L-rhamno-beta-D-glucopyranoside (m/z 598.18977), naringenin-7-o-glucoside (m/z 434.12129), epicatechin (m/z 290.07903), proanthocyanidin b1 (m/z 578.14242), chlorogenic acid (m/z 354.09508), bergenin (m/z 328.07943), leucocynidin (m/z 306.07395), gallic acid (m/z 170.02152), mesoinositol (m/z 180.06338), caffeic acid (m/z 180.04225), and catechin (m/z 290.07903), respectively [Table 1 and Figure 1].

Aqueous extract of Panchavalkala altered the viability of cervical cancer cells

The effect of PVaq was evaluated on the viability of SiHa and HeLa cells by plating them and allowing them to grow until they reached confluency. These cells were then cultured in the presence of various concentrations (0–320 μg/ml) of the extract for 24 h. There was significant reduction ($P < 0.001$) in the viability of SiHa (75.2% ± 12.8%) and HeLa (75.03% ± 6.06%) cells at 80 μg/ml concentration of PVaq compared to the untreated control cells [Figure 2]. IC_{50} of PVaq for SiHa and HeLa was found to be 125.8 and 96.0 μg/ml, respectively.

Aqueous extract of Panchavalkala altered the growth kinetics of cancer cells

To test the effect of PVaq on the growth kinetics of SiHa and HeLa, the cells were treated with different concentrations (0, 20, 40, and 80 μg/ml) of the extract and grown for 24, 48, and 72 h. At the end of each treatment, the cells were stained with trypan blue and the number of viable cells was counted. It was observed that, compared to the control (untreated) cells, PVaq significantly ($P < 0.001$, $n = 6$) reduced the growth of SiHa and HeLa at all the tested concentrations. At the maximum concentration of 80 μg/ml, PVaq reduced the growth of SiHa by 67.4% ± 0.25%, 91.9% ± 0.1%, and 92.8% ± 0.18% at 24, 48, and 72 h, respectively [Figure 3a]. On the other hand, in HeLa, PVaq reduced the growth at 80 μg/ml by 81.7% ± 0.2%, 99.6% ± 0.05%, and 88.23% ± 0.17% at 24, 48, 72 h, respectively [Figure 3b]. This was further supported by soft agar assay wherein a dose-dependent decrease in the number of colonies was observed in both the cervical cancer cell lines [Figure 3c]. Interestingly, at 80 μg/ml concentration, PVaq significantly reduced the number of colonies in SiHa (86.9% ± 1.1%) and HeLa (82.4% ± 1.2%) compared to their respective untreated control cells [Figure 3c]. These results showed that PVaq regulated the growth of cervical cancer cells in a significant manner.

DISCUSSION

Despite therapeutic advances in cancer, the associated adverse events and chemoresistance remain major challenges to be addressed. During the past two decades, there have been extensive studies on the use of medicinal plants or their phytoconstituents as promising chemopreventive as well as anticancer agents. More than 60% of currently used anticancer drugs are originally derived from natural sources such as plants, marine organisms, and microorganisms.[18,19]

Traditional medicine that includes herbal-based drugs have been used worldwide from a long time to treat various chronic ailments including cancer. Various scientific studies, including ours, have suggested the potential of medicinal plants as anticancer drug candidates.[11,16,17] Ayurveda, the main traditional medical practice in India, primarily deals with the prevention of disease through healthy food habits and lifestyle.[15,16]It has been successful from very early times in using natural drugs and preventing or suppressing various tumors using various lines of treatment.[20,21] Charak Samhita (1500–2000 BC), the supposed first text of Ayurveda, reports various formulations in the form of kashya (decoctions), each formulation containing different herbs that target a specific action.[22]

Panchavalkala is a well- known Ayurvedic polyherbal formulation that has been reported to be used against inflammation, to clear ulcers, dress wounds, as a douche in leukorrhea and other vaginal diseases.[6] The free radical scavenging activity of Panchavalkala and its individual components has been reported.[23] Panchavalkala has been reported to be used as an adjunct in the treatment of leukorrhea.[7] The bark of the individual components of Panchavalkala which include *T. populnea* Solandexcorea,[24,25] *F. benghalensis* L,[26,27] and *F. religiosa L*[28] have been proven to possess antioxidant and anti-inflammatory activities. The present work reports for the first time the anticancer activity of Panchavalkala in cervical cancer.

PVaq showed presence of around 77 phytocompounds. Behenic acid is a fatty acid; vogelin is an isoflavone; and leucocyanidin is a flavonoid with no reported pharmacological properties. Acacetin is a flavonoid with anti-inflammatory[29] and anticancer properties.[30] Leucopelargonidin 3-O-alpha-L-rhamno-beta-D-glucopyranoside is a flavonoid with reported antidiabetic property.[31] Naringenin-7-O-Glucoside, epicatechin, and proanthocyanidin B1 are flavonoids with reported antioxidant[32-34] and antitumor[35-37] properties. Bergenin is a glycoside with antihepatotoxic,[38] antiulcerogenic, anti-HIV, antifungal, hepatoprotective, antiarrhythmic, neuroprotective, anti-inflammatory, immunomodulatory, and burn wound healing properties.[39] Chlorogenic

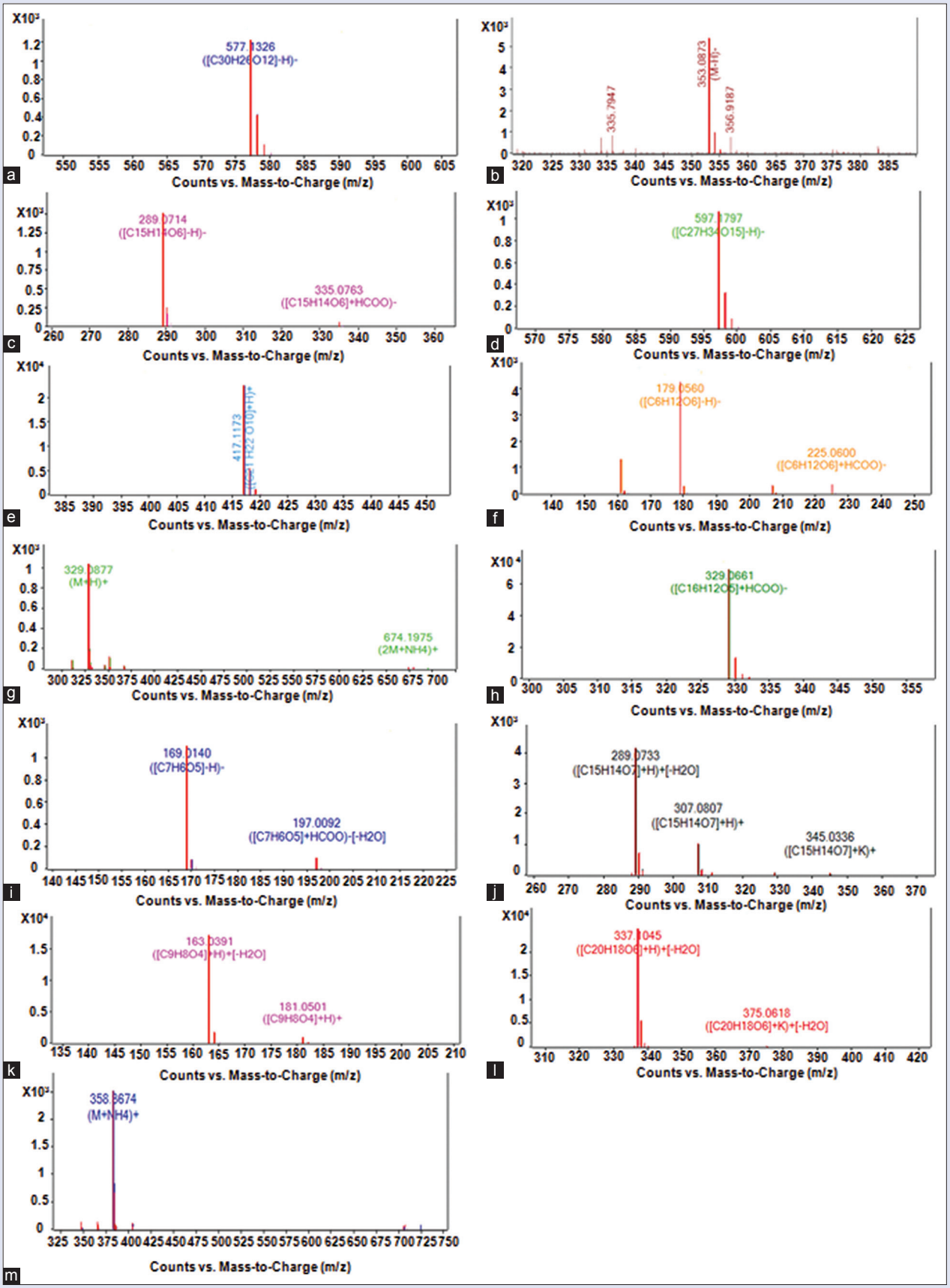

Figure 1: Liquid chromatography–mass spectrometry/mass spectrometry pattern of important compounds identified in aqueous extract of panchavalkala. Proanthocyanidin B1 (a), chlorogenic acid (b), epicatechin/Catechin (c), leucopelargonidin 3-O-alpha-L-rhamno-beta-D-glucopyranoside (d), naringenin-7-O-Glucoside (e), mesoinositol (f), bergenin (g), acacetin (h), gallic acid (i), leucocynidin (j), caffeic acid (k), vogelin E (l), behenic acid (m)

acid is a bioflavonoid which exhibits pharmacological activity such as antioxidant,[40] antidiabetic,[41] and antiobesity.[41] Gallic acid is a phenol with antiviral,[42] anti-inflammatory,[43] anticancer,[44] and antidiabetic[45] properties. Mesoinositol is a sugar alcohol with no reported pharmacological activity. Caffeic acid and catechin are polyphenols with anti-inflammatory,[46] anticancer[47,48] and antiviral[49,50] properties.

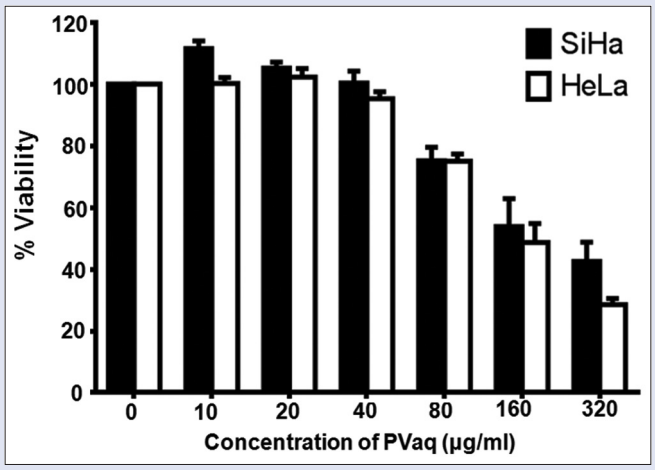

Figure 2: Effect of aqueous extract of Panchavalkala on cell viability in SiHa and HeLa. The cells were treated with different concentrations (0–320 µg/ml) of aqueous extract of Panchavalkala for 24 h. The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The presence of proanthocyanidin B1, chlorogenic acid, caffeic, and epicatechin acid present in PVaq could be contributed to the components of *F. religiosa* as reported earlier.[25] The presence of leucopelargonidin 3-o-alpha-l-rhamno-beta-d-glucopyranoside, leucocynidin, naringenin-7-o-glucoside, and mesoinositol could be contributed to the phytocompounds reported in the bark extract of *F. benghalensis*.[51-55] The phytochemicals catechin, vogelin E, and mesoinositol found in PVaq could be contributed to the reported compounds of *F. virens*.[56,57] The The compounds behenic acid and bergeninin PVaq have been reported in the bark extract of *F. glomerata*.[58] The phytocompounds acacetin and and gallic acid in PVaq have been reported in *T. populnea*.[59-61] Thus, these data show the presence of marker compounds in the PVaq that could be used for confirming the authenticity of the formulation to avoid batch-to-batch variation.

Interestingly, PVaq exhibited anticancer activity against HPV-positive cervical cancer cell lines wherein it decreased the viability of the cells. Moreover, PVaq reduced the growth rate of cells in a time- and dose-dependent manner. The anticancer activity of PVaq could be attributed to the presence of different phytocompounds in the extract with reported anticancer activity. Proanthocyanidins,[62] catechin,[63,64] naringenin-7-o-glucoside,[65] and acacetin[66] have been reported to suppress the growth of breast cancer cells. Mesoinositol also hasreported anticancer activity.[67] We have previously reported that *F. religiosa*,

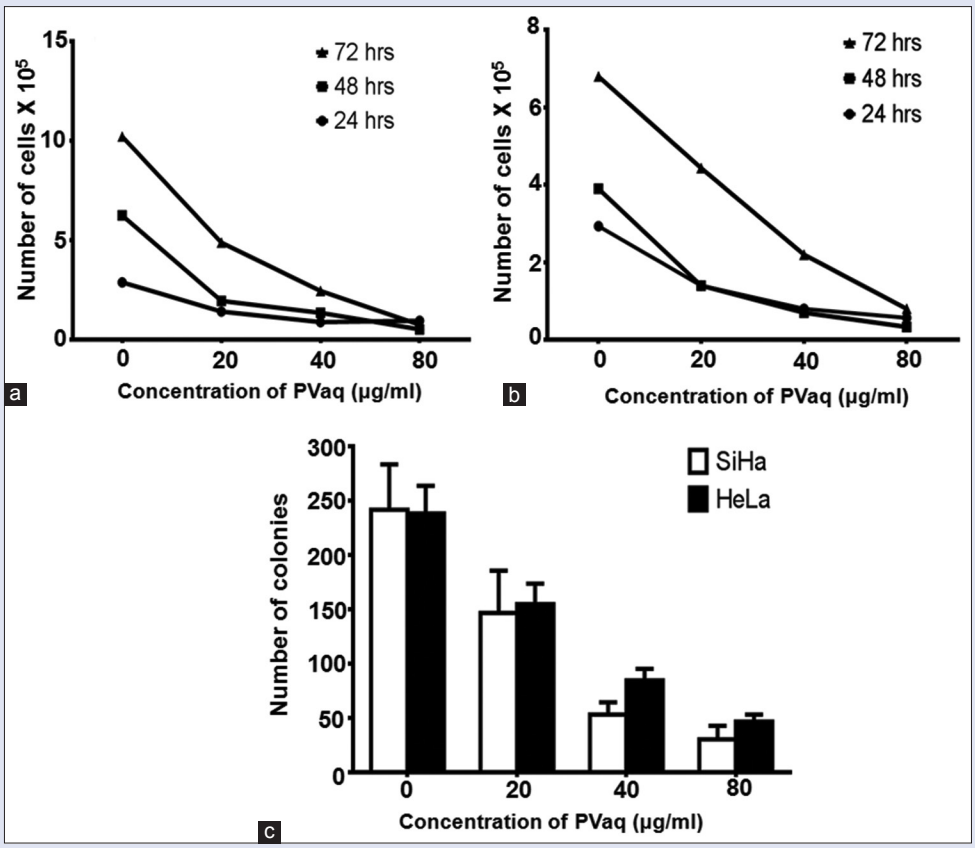

Figure 3: Effect of aqueous extract of Panchavalkala on growth kinetics of cervical cancer cells. SiHa (a) and HeLa (b) cells were treated with aqueous extract of Panchavalkala (0–80 µg/ml) for 24–72 h and the number of viable cells were counted using the trypan blue dye exclusion method. Data represent mean ± standard deviation of three independent experiments. (c) The cervical cancer cell lines (SiHa and HeLa) (5×10^3) along with aqueous extract of Panchavalkala (0–80 µg/ml) were grown in soft agar for 2 weeks. Colonies were counted from at least ten different areas and the average of each has been plotted. The data represent mean ± standard deviation of three independent experiments

one of the components of PVaq, induced cell cycle arrest in SiHa and apoptosis in HeLa and apoptosis in HeLa (HPV-18 positive) cells. These preliminary data warrant future in-depth studies at molecular and *in vivo* levels to delineate the mechanism of anticancer activity of Panchavalkala in detail.

CONCLUSION

Panchavalkala, an Ayurvedic formulation, was reported to exhibit anticancer activity against HPV-positive cervical cancer cell lines. The phytochemical evaluation of the PVaq has shown the presence of phytochemicals that have reported anticancer activity, thereby signifying the importance of this formulation as a prospective drug candidate in the management of cervical cancer. However, detailed experimentation is required in the future for understanding the underlying mechanism of its action.

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Conflicts of interest

There are no conflicts of interest.

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