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ORIGINAL ARTICLE

In vitro Antioxidant and Anticancer Activities of Leaf Extracts of *Rhododendron arboreum* and *Rhododendron campanulatum* from Uttarakhand Region of India

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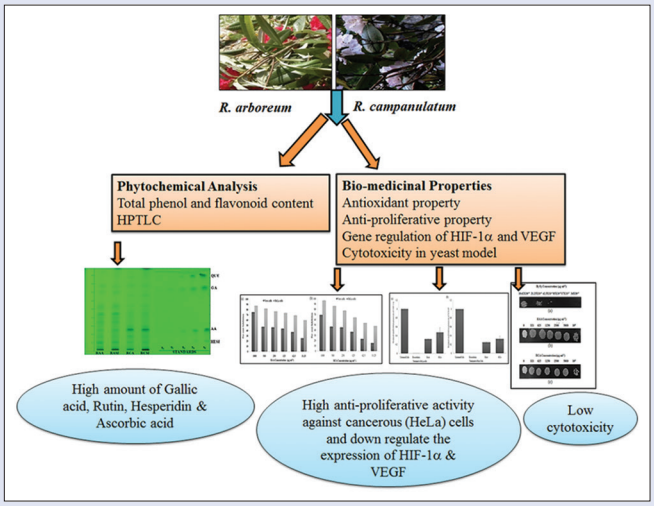
ABSTRACT

Background: *Rhododendron arboreum* and *Rhododendron campanulatum* are two important trees of Uttarakhand, known for their ethnopharmacological importance. In the present study, the aqueous and methanolic extracts of leaves of both the plants were analyzed for phytochemical, antioxidant, and antiproliferative activities against cancer cell line and repression of vascular endothelial cell growth factor (VEGF) and hypoxia-inducible factor-1 (HIF-1α) transcription. **Materials and Methods:** Aqueous and methanolic leaf extracts of both the plants were prepared through pressurized liquid extraction method. Various assays were performed to analyze phytochemical and antioxidant potential. The antiproliferative activity was determined through 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxy anilide salt (XTT) assay. Further, the effect of plant extracts on the modulation of HIF-1α and VEGF mRNA expression was analyzed through quantitative real-time polymerase chain reaction. **Results:** A significantly high total phenol content, total flavonoid content, free radical scavenging, and reducing power activities were observed in both the plant extracts. High-performance thin-layer chromatography analysis indicated the presence of ascorbic acid, quercetin, gallic acid, and hesperidin in different extracts. XTT reduction assay confirmed the antiproliferative activities of aqueous extracts against cancer cell line, HeLa. Aqueous extract of *R. arboreum* (RAA), at a concentration of 31.25 μg/ml, inhibited 60.12% and 25.41% proliferation of HeLa and Vero cell lines, respectively. An equal concentration of aqueous extract of *R. campanulatum* (RCA) inhibited the growth by 48.04% and 15.17% for HeLa and Vero cell lines, respectively. Further, the RAA and RCA have downregulated the expression of two key angiogenic factors responsible for tumor neovascularization, HIF-1α and VEGF to a great extent. **Conclusion:** The present study affirms that the leaf extracts of *R. arboreum* and *R. campanulatum* have enormous potential to be developed as an effective natural antioxidant and anticancer drug.

Key words: Anticancer, hypoxia-inducible factor-1, HeLa, *Rhododendron arboreum*, *Rhododendron campanulatum,* vascular endothelial cell growth factor

SUMMARY

- The methanolic and aqueous extracts of leaves of Rhododendron arboreum and R. campanulatum exhibited good antioxidant properties which correspond to their phenolic and flavonoid contents.
- The aqueous extracts have shown promising anti-proliferative activities against cancerous (HeLa) cell line which was higher than the cytotoxicity against normal (Vero) cell line.
- The aqueous extracts have down- regulated the mRNA levels of oncogenic factors viz. HIF-1 alpha and VEGF in HeLa cells.



Abbreviations used: PLE: Pressurized liquid extraction; XTT: 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxy anilide salt; TPC: Total phenol content; TFC: Total flavonoid content.

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INTRODUCTION

Cancer is associated with the highest mortality after cardiovascular disorders worldwide.[1] There will be around 17 million cancer deaths per year and 26 million new tumor cases around the world, by 2030.[2] Cancer is associated with lethal clinical manifestations, characterized by abnormal cell proliferation, defective DNA replication, checkpoints, and apoptotic pathways.[3] Radiotherapy, surgery, and chemotherapy are main antitumor and anticancer therapies, but these possess several side

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effects and lesser survivable rate in a patient, giving rise to need of new anticancer drugs of natural origin.[4,5] Exposure to radiations is common to healthcare professionals and patients undergoing radiotherapy or radiodiagnosis, which may lead to generation of free radicals causing DNA lesions and ultimately genome instability, leading to cancer.[6] Free radicals are unstable reactive molecules, which are responsible for extensive intracellular damage, especially to proteins, DNA, and lipids, leading to several diseases and disorders such as cancer, diabetes, neurological disorders, pulmonary diseases, and cardiovascular diseases.[7,8]

Vascular endothelial cell growth factor (VEGF) is an endothelial-specific mitogen that stimulates angiogenesis.[9] VEGF has been validated as a therapeutic target in many types of human cancers.[10] Hypoxia-inducible factor-1 (HIF-1), a transcription factor, regulates the expression of VEGF in the hypoxic microenvironment of the tumors.[11] Upregulation of HIF-1 and VEGF expression has been reported in many types of human cancer.[12] HIF-1 consists of two subunits HIF-1α (regulatory subunit) and HIF-1β (ubiquitously expressed subunit). HIF-1α is upregulated in cancerous cells and results in aggressiveness of tumor cell behavior, angiogenesis, and metastasis.[13-15] HIF-1 and VEGF pathways are the major targets for anticancer drugs as they play a crucial role in the activation of severe pathological conditions in cancer patients.[16,17] Many plant-based natural products have been reported to repress the expression of HIF-1α and VEGF.[18,19]

From the prehistoric time, natural products and their derivatives have been used as anticancer drugs and antioxidants.[20] The National Cancer Institute (NCI) had screened about 114,000 plant extracts for their anticancer properties, derived from 35,000 plant samples collected from 20 countries.[21] Plant-derived agents such as topotecan, taxol, docetaxel, camptothecin, etoposide, and vinca alkaloids are used clinically against various types of cancers.[22] *Catharanthus roseus*, *Taxus baccata* L., *Viscum album* L., *Panax ginseng*, and *Rhododendron* sp. are some medicinally important plants which exhibited anticancer and antioxidant properties.[22-27]

More than 800 species of *Rhododendron* (Family: *Ericaceae*) are reported worldwide; many of them have ethnopharmacological importance.[28] *Rhododendron arboreum* Sm. and *Rhododendron campanulatum* D. Don are two major species found in Uttarakhand region, India. *R. arboreum* Sm. is a state tree of Uttarakhand and juice of its flowers is consumed as adaptogenic seasonal drink in Uttarakhand due to the presence of several phytoconstituents such as epicatechin, quercetin, syringic acid, rutin, quercetin, and coumaric acid.[29-31] *R. arboreum* is reported to possess several medicinal and pharmacological properties such as hepatoprotective, antioxidant, immunomodulatory, anti-inflammatory, antidiabetic, and antinociceptive.[32-35] *R. campanulatum* D. Don is an evergreen gregarious shrub or a dwarf tree found in the inner and outer ranges of the Alpine Himalayas.[36,37] The plant was reported to have several phytoconstituents such as quercetin, protocatechuic acid, chlorogenic acid, gallic acid, epicatechin, and oleanane triterpene.[30,38,39] Conventionally, the leaves of *R. campanulatum* are used in sciatic, syphilis, skin diseases, throat pain and body ache, chronic fevers, and chronic rheumatism.[28,30] Aerial parts of *R. campanulatum* have been reported to possess antioxidant, antimicrobial, and immunomodulatory activities.[33,40-42]

In the present study, we studied the phytochemical, *in vitro* antioxidant, and anticancer properties of the leaf extracts of *R. arboreum* Sm. and *R. campanulatum* D. Don from the Indian Himalayan region of Uttarakhand. The extracts were prepared through pressurized liquid extraction (PLE) method with an aim to enrich the extracts with phenolics.[43] The two plant extracts were investigated for antiproliferative properties against cervical cancer cell line, HeLa and noncancerous cell line, Vero, and the effect of the extracts on the expression of HIF-1α and VEGF was analyzed in HeLa cell line.

MATERIALS AND METHODS

Reagents, chemicals, and culture conditions

2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxy anilide salt (XTT), menadione, RPMI 1640 medium, and Trolox were procured from HiMedia (Mumbai, Maharashtra, India). All other reagents and chemicals used were of analytical grade and were purchased from Sigma Chemicals (St. Louis, MO, USA), Merck Co. (Darmstadt, Germany), and Fischer Scientific (Mumbai, Maharashtra, India).

Vero (African Green Monkey Kidney; normal cell line) and HeLa (cervical adenocarcinoma cell line) cell lines were procured from National Centre for Cell Sciences, Pune, Maharashtra, India, for antiproliferative and gene expression experiments. The cells were maintained and continuously sub cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, amphotericin B (2.5 μg/ml), and gentamicin (50 μg/ml) at 37°C and 5% CO_2.

Cytotoxicity of aqueous extract of *R. arboreum* (RAA) and aqueous extract of *R. campanulatum* (RCA) was studied in yeast model (*Saccharomyces cerevisiae*; strain BY4741) by spot assay. Yeast cells were maintained and grown in yeast extract peptone dextrose (YPD) medium (2% glucose, 2% Bacto peptone, and 1% yeast extract). The spot assay was performed in microtiter plate and yeast culture was grown in RPMI-1640 medium.

Collection of plant material and preparation of extracts

The leaves of *R. arboreum* were collected from Chourangi region of Uttarkashi district (1352 m asl) and *R. campanulatum* was collected from the forest of Govindghat of Chamoli district (3348 m asl) in May 2013. Plant samples were identified in the Department of Botany and Microbiology at Hemwati Nandan Bahuguna Garhwal University, Srinagar, Uttarakhand, and the specimen was deposited in the Departmental Herbarium with the voucher numbers GUH 20742 and GUH 20743 to *R. arboreum* and *R. campanulatum*, respectively.

One hundred grams (g) freshly collected leaves of each plant was rinsed and shade-dried to 45 g and 58 g for *R. arboreum* and *R. campanulatum*, respectively. The dried leaves were coarsely powdered using an electric blender and subjected to PLE in Accelerated Solvent Extractor System (ASE350, Dionex Corporation, Sunnyvale, CA, USA), equipped with a solvent controller unit under a high pressure of about 1500 psi.[44] Aqueous and methanolic extracts of R. arboreum (henceforth named as RAA and RAM, respectively) and R. campanulatum (henceforth named as RCA and RCM, respectively) were lyophilized to powder form and stored at 4 °C till further use.

Phytochemical analyses

Estimation of total phenol and total flavonoid content

Folin–Ciocalteu reagent (FCR) assay was performed for the estimation of total phenolic content (TPC) of the extracts.[45,46] One hundred and fifty microliters of the extract (1 mg/ml) or standard was mixed with 240 μl of water and 150 μl of 0.25 N FCR and incubated for 3 min in the dark at room temperature. After incubation, 300 μl of 1 N Na_2CO_3 was added, and the mixture was further incubated for 2 h in the dark at room temperature. Different dilutions of gallic acid (0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.08 mg/ml, and 0.1 mg/ml) were used as a standard for drawing the calibration curve. The absorbance of each sample was measured at 765 nm and the results were expressed in terms of mg of gallic acid equivalent/g of the extract.

Total flavonoid content (TFC) assay was performed as suggested by Kim *et al.*, with slight modifications.[47] In 10 ml of extract (1 mg/ml) or standard, 2 ml of water and 0.15 ml of 5% $NaNO_2$ solution were added. The mixture was allowed to react for 6 min and 0.15 ml of 10% $AlCl_3$ solution was added to it. The absorbance was measured at 510 nm after 15 min. Different dilutions of rutin (0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.08 mg/ml, and 0.1 mg/ml) were taken as standard. All values were expressed in terms of mg of rutin equivalent per gram of extract.

High-performance thin-layer chromatography analysis

All extracts were analyzed for the presence of selected phytochemicals through high-performance thin-layer chromatography (HPTLC).[48] Gallic acid, ascorbic acid, hesperidin, and quercetin were used as standards. A stock solution of 1 mg/ml was prepared of each standard in methanol and 20 µl of each containing different amounts of the standard (S_1: 0.20 µg, S_2: 0.40 µg, S_3: 0.80 µg, S_4: 2.00 µg, S_5: 4.00 µg) was used for spotting. Twenty milligrams of each sample was dissolved in 1 ml methanol.

For HPTLC analysis, 20 µl of each test and standard solution were loaded as 6 mm band length in the 20 cm × 20 cm Silica gel 60 F_{254} plates (0.2 mm thick) using CAMAG Linomat 4 applicator, controlled through Automatic LC sampler 4 (ATS4, CAMAG, Muttenz, Switzerland). TLC plates were developed in CAMAG twin trough development chamber (20 cm × 20 cm) which was presaturated with mobile phase (ethyl acetate: dichloromethane: glacial acetic acid: formic acid: methanol [5/5/0.5/0.5/1 v/v]) for 20 min at room temperature (25°C ± 2°C). After development, plates were dried for 15 min and then documented through Reprostar 3 documentation system using illumination at 254 nm and 366 nm. Quantification was performed with CAMAG Scanner III at a wavelength of 254 nm with following conditions: slit dimension 4.00 mm × 0.30 mm, scanning speed 20 mm/s, and data resolution 100 µm/step. To identify the phytochemical in bands, ultraviolet (UV) absorption spectrum of each standard was overlaid with the corresponding band (on the basis of R_f values) in the sample track. For quantification, retention factor (R_f) and area under curve (AUC) were analyzed with WinCATS Planar Chromatography Manager software (version: 1.4.4.6337; CAMAG (Muttenz, Switzerland) system). Calibration curve was prepared for all the standards using five dilutions of the standards. Experiments were repeated at least three times and the best representative of the results is shown.

Antioxidant assay

1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay was performed following Brand-Williams *et al.*[49] The DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol and is stored at −20°C until needed. The working solution was prepared by mixing 10 ml stock solution in 45 ml methanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. One hundred and fifty microliters of extract (1 mg/ml) or standard solutions (0.01 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.08 mg/ml, and 0.1 mg/ml) were allowed to react with 2850 µl of the DPPH solution for 2 h in dark. Finally, the absorbance was taken at 515 nm and the scavenging activity was calculated. Trolox was used as a standard at different concentrations. Negative control (without standard/extract) sample was also included in the study. Percent DPPH-free radical scavenging activity is calculated by the formula written below:

Scavenging activity (%) = $(A_{control} - A_{extract})/A_{control} \times 100$

Where $A_{control}$: Absorbance of control; $A_{extract}$: Absorbance of extract.

2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging assay

2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) scavenging assay was performed following Thaipong *et al.*[50] with slight modifications. The stock ABTS solution was prepared by adding 7.4 mM ABTS to 2.6 mM potassium persulfate in the ratio of 1:1 and is incubated overnight in dark at room temperature to produce ABTS radical (solution turns to blue in color). Further, the working solution was prepared by mixing 1 ml of stock ABTS solution with 60 ml of methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm. Two hundred and fifty microliters of ABTS radical (working solution) was added to 10 µl of each extract (1 mg/ml) and standard solutions of Trolox (0.01, 0.02, 0.04, 0.08, and 0.1 mg/ml) in a microtiter plate. Absorbances were taken at 734 nm after 15 min incubation. The $ABTS^+$ radical without extract and standard was used as control. Results were measured as percent ABTS-free radical scavenging activity. The percent scavenging rate is calculated using the following formula:

Scavenging activity (%) = $(A_{control} - A_{extract})/A_{control} \times 100$

Where $A_{control}$: Absorbance of control; $A_{extract}$: Absorbance of extract.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay was performed to calculate total antioxidant power of the extract.[51] The FRAP solution was freshly prepared by mixing 25 ml of 30 mM acetate buffer, 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine, and 2.5 ml of 20 mM $FeCl_3.6H_2O$ solution. One hundred and fifty microliters of each plant extract (1 mg/ml) was allowed to react with 2850 µl of the freshly prepared FRAP solution. The solution was incubated in the dark for 30 min. The absorbance was measured at 593 nm. Results were expressed in µg Trolox equivalent/g (µg TE/g) of extract, using a standard curve prepared from different concentration of Trolox.[52,53]

Total reducing power assay

Total reducing power (TRP) assay was performed as described by Li *et al.*[54] One milliliter of extract (1 mg/ml) or ascorbic acid (0.025 mg/ml, 0.050 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, and 0.25 mg/ml) solution was added to 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferrocyanide. This solution was allowed to stand for 20 min in water bath at 50°C. Afterward, 2.5 ml of 10% trichloroacetic acid was added to solution. Then, it is centrifuged at 3000 rpm for 10 min. Then, 2.5 ml aliquot of upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The solution was mixed thoroughly and the absorbance of the solution was recorded at 700 nm. Ascorbic acid at different concentrations (mg/ml) was taken as standard and the results were expressed in µg ascorbic acid equivalent/g (µg AE/g) of the extract.[55,56]

In vitro anticancer activity

Antiproliferative activity

The antiproliferative activity was evaluated by XTT reduction assay.[57]

1×10^5 cells were seeded in each well of a 96-well microtiter plate containing DMEM and plate was incubated for 24 h at 37°C with 5% of CO_2 to get the wells 80%–90% confluent with the cells. Only aqueous extracts (RAA and RCA) were used in the assay. Fresh DMEM containing extracts (RAA or RCA) in decreasing log_2 concentration (1000 µg/ml–31.2 µg/ml) were dispensed in each well and the plate was incubated for 48 h at 37°C with 5% of CO_2. After 48 h incubation, the cells were washed with phosphate-buffered saline, and 40 µL of XTT and 2 µL of 50 µM menadione reagent were added to each well. The plate was further incubated for another 2 h, and absorbance was measured

at 490 nm in microtiter plate reader (Bio-Rad). All experiments were performed in triplicate, and the results are shown in the form of the % inhibition of cell lines used. Following is the formula for the calculation of % inhibition.

% Cell inhibition = 100 − % cell viability

Mean values of the % inhibition ± standard deviation (SD) are used for plotting the bar graphs at each concentration.

Expression of hypoxia-inducible factor-1α and vascular endothelial cell growth factor mRNA level

The effect of plant extracts on the modulation of HIF-1α and VEGF mRNA expression was analyzed through quantitative real-time polymerase chain reaction (RT-PCR).[58] The 18srRNA gene was used as an internal control. Doxorubicin, a standard anticancer drug, was used as positive control in the present study.[59]

Drug treatment and RNA extraction

HeLa (cervical adenocarcinoma) cells were maintained in DMEM and further subcultured in 90 mm Petri dishes in an adherent manner. Cells in separate Petri dishes were treated with either 500 µg/ml of extracts (RAA or RCA) or 5 µg/ml of positive drug control (doxorubicin), along with one untreated negative control. The cells were incubated with samples or control for 24 h before RNA isolation. Morphology of the cells under different treatments was analyzed, using inverted light microscope (LEADZ Trinocular microscope) at ×10. After 24 h of treatment, total RNA was extracted from HeLa cell lines using RNASure® Mini Kit (Nucleo-pore™, Catalogue No-NP-84105) following the manufacturer's instructions. Further, RNA samples were quantified using NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 2000C), and the purity was ascertained by $A_{260/280}$ ratios. The intactness of RNA samples was checked by the visualizing 28srRNA and 18srRNA bands through agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse-Transcription Kit of Applied Biosystems (Catalog no. 43-688-14), following the manufacturer's instruction.

Real-time polymerase chain reaction

To quantify the expression of HIF1α and VEGF, quantitative RT-PCR was performed using cDNA products as a template in Applied Biosystems 7500 Fast RT-PCR System, using SYBR® Green PCR Master Mix (Catalog no. 4309155) of the Applied Biosystems. Gene-specific primers were used for amplification [Table 1]. 18srRNA was used as control and each reaction was repeated three times.

Amplification was carried out with an initial incubation at 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min, for a total of 40 cycles, and 60°C for 10 min. Data analysis was carried out using 7500 Sequence Detection Software v2.0.6 (Applied Biosystems 7500 Real-Time PCR System) and the fold change ($2^{-\Delta\Delta Ct}$) was measured, based upon threshold cycle values.[60]

Cytotoxicity in yeast model

Cytotoxicity of RAA and RCA were studied in yeast model (*S. cerevisiae*; strain BY4741) through spotting assay.[61,62] One hundred microliters RPMI having 1×10^3 log phase cells of BY4741 ($OD_{600\text{-}0.6}$) was added to each well of a round bottom microtiter plate. Further, 100 µl of different dilutions of extracts in RPMI (in increasing $\log_2$ concentration: 320–10,000 µg/ml) or dilutions of H_2O_2 in RPMI (in increasing $\log_2$ concentration: 0.0010625–0.034 µg/ml) was added to separate wells. Microtiter plates were incubated for 16 h at 28°C with shaking. After the incubation, 5 µl of culture from each well was spotted onto YPD agar plates in triplicates and the plates were incubated for 24 h at 28°C before being photographed.

Statistical analysis

All the experiments were repeated at least in triplicates. The experimental data were expressed as mean ± SD. Significant differences between two data series are calculated through Student's *t*-test. The results were analyzed using Student's *t*-test to determine the level of significance, and the level of significance was set at $P < 0.05$.

RESULTS

PLE resulted in the yield of 19% for RAM, 12.2% of RCM, 16.2% of RAA, and 11.8% of RCA.

Phytochemical analyses

Estimation of total phenol and total flavonoid content

As given in Table 2, extracts were found to have good TPC and TFC values. TPCs of all four extracts did not differ much, while TFC values ranged from 503.97 ± 1.71 to 970.91 ± 0.47 mg RE/g for the extracts. RAA showed the highest flavonoid content followed by RCA, RAM, and RCM [Table 2].

High-performance thin-layer chromatography analysis

The method was validated for specificity and linearity. The optimized solvent system gave the best resolution with symmetric and reproducible peaks, confirmed by specific Rf values for each standard (ascorbic acid: 0.31, gallic acid: 0.74, hesperidin: 0.20, and quercetin: 0.87). The plates were visualized under UV light at 254 nm and 366 nm for

Table 1: Primers used in the study

Name of primer	Sequence
HIF-1α forward	5'-GAGATGTTAGCTCCCTATATCCCA-3'
HIF-1α reverse	5'-TAGGTTCTTGTATTTGAGTCTGCTG-3'
VEGF forward	5'-TACTGCCATCCAATCGAGAC-3'
VEGF reverse	5'-GCATGGTTGATGTTGGACT-3'
18srRNA forward	5'-TCGGAACTGAGGCCT-3'
18srRNA reverse	5-CTTTCGCTCTGGTCCGTCTT-3'

VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia-inducible factor-1

Table 2: Total phenolic content and total flavonoid content of the extracts

Plant extracts	Total phenolic content mg GAE/g of extract±SD	Total flavonoid content mg RE/g of extract±SD
RAA	101.64±0.013	970.91±0.47
RAM	102.7±0.017	651.02±1.42
RCA	102.5±0.011	527.84±1.90
RCM	102.7±0.014	503.97±1.71

RAA: Aqueous extract of *Rhododendron arboreum*; RAM: Methanolic extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*; RCM: Methanolic extract of *Rhododendron campanulatum*; SD: Standard deviation

Table 3: Quantification of phytochemicals in the extracts by high-performance thin-layer chromatography

Samples	Ascorbic acid (ng/µg extract)	Gallic acid (ng/µg extract)	Hesperidin (ng/µg extract)	Quercetin (ng/µg extract)
RAA	15.63	2.36	8.45	1.89
RAM	1.805	3.15	9.35	BDL
RCA	3.29	5.74	BDL	2.22
RCM	2.58	3.02	BDL	BDL

RAA: Aqueous extract of *Rhododendron arboreum*; RAM: Methanolic extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*; RCM: Methanolic extract of *Rhododendron campanulatum*; BDL: Below detection limit

generating fingerprint of phytoconstituents [Figure 1]. Densitometric chromatogram indicated the presence of ascorbic acid, gallic acid, hesperidin, and quercetin in different concentrations in the RAA, RCA, RCA, and RCM [Figure 2]. Ascorbic acid, gallic acid, hesperidin, and quercetin were quantified in RAA, RCA, RCA, and RCM [Table 3]. Hesperidin was not detected in RCA and RCM and quercetin was not detected in RAM and RCM.

Antioxidant assay

Free radical scavenging activity of extracts was analyzed through DPPH and ABTS scavenging assays. FRAP and TRP assays were used for analyzing reducing powers of the extracts.

1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay

Trolox was used to plot calibration curve, generating linear equation, $Y = -2.790X + 1.483$, and regression coefficient, $R^2 = 0.98$. RAM showed the highest radical scavenging activity (91.67% ±0.140%), followed by RCM, RAA, and RCA with marginal differences [Table 4].

2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt assay

Trolox was used to draw calibration curve, characterized with linear equation, $Y = -3.279X + 1.293$, and regression coefficient, $R^2 = 0.973$. The extracts showed almost similar ABTS scavenging activities, ranging from 96.48% to 96.65% [Table 4]. Aqueous extracts of two plants have shown significant differences in ABTS scavenging activities ($P < 0.05$).

Ferric reducing antioxidant power assay

FRAP is a simple and direct method to measure antioxidant activity, where antioxidants reduce ferric ions into ferrous ions and can be detected at 593 nm wavelength. Trolox was used to draw calibration curve, characterized with linear equation, $Y = 6.556X + 0.117$, and regression coefficient, $R^2 = 0.981$. As shown in Table 4, RAM showed the highest reducing power activity (429.07 ± 0.019 µg TE/g) among all. *R. arboreum* extracts showed better FRAP activities than *R. campanulatum* extracts.

Total reducing power assay

TRP assay measures the electron donating capacity of antioxidant and higher absorbance indicates the higher reducing power.[63] In TRP assay, ascorbic acid is used as a standard and calibration curve was plotted ($Y = 4.124X + 0.070$, $R^2 = 0.979$). As per Table 4, TPRs of the extracts ranged from 271.55 ± 0.015 µg AE g^{-1} to 292.27 ± 0.020 µg AE g^{-1} (for RAM).

In vitro anticancer activity

Antiproliferative assay

Only aqueous extracts were used in this study. As shown in Figure 3, RAA and RCA inhibited HeLa cells better than Vero cells. Dose-dependent inhibition by RAA ranged from 75.30% to 25.41% and 87.66% to 60.12% for Vero and HeLa cell lines, respectively [Figure 3]. Similarly, RCA showed the inhibitions ranging from 69.08% to 15.17% and 96.74% to 48.04% for Vero and HeLa cell lines, respectively.

Inhibition was higher in cancer cell line (HeLa) than the inhibition in normal cell line (Vero), suggesting the lesser side effect on normal cells.

Expression of hypoxia-inducible factor-1α and vascular endothelial cell growth factor mRNA levels

We investigated the effect of RAA and RCA on the expression of HIF-1α and VEGF mRNAs levels in HeLa cells. Cells were photographed at ×10 through inverted light microscope after 24 h and exposure to positive control and the extracts. HeLa cells treated with doxorubicin or RAA or RCA showed cytoplasmic shrinkage, chromatin compaction, and reduction in adherence [Figure 4]. Cells treated with doxorubicin showed the highest deterioration, followed by RAA and then RCA, as shown in Figure 4.

The fold changes (with respect to the untreated control) in the mRNA levels of HIF-1α and VEGF were analyzed in the presence and absence of the extracts and doxorubicin through quantitative RT-PCR. At the given conditions, an exposure of 500 µg/ml RAA for 24 h has downregulated the expression of HIF-1α and VEGF to 0.322 folds and

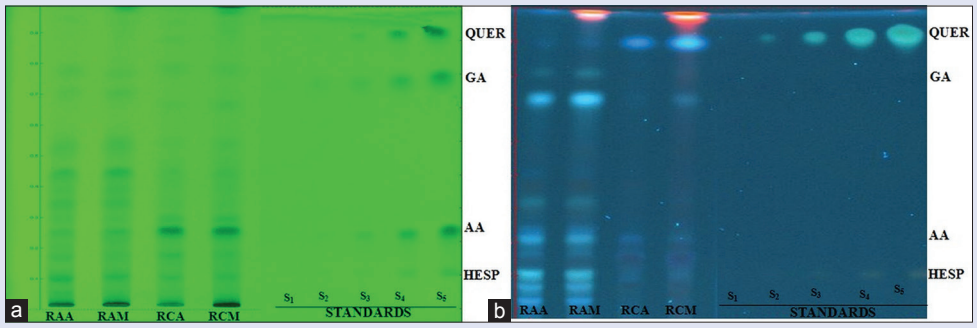

Figure 1: High-performance thin-layer chromatograms (a) at 254 nm and (b) at 366 nm. RAA: Aqueous extract of *Rhododendron arboreum*; RAM: Methanolic extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*; RCM: Methanolic extract of *Rhododendron campanulatum*; QUER: Quercetin; GA: Gallic acid; AA: Ascorbic acid; HESP: Hesperidin

Table 4: *In vitro* antioxidant activities of the extracts

Plant extract	DPPH scavenging activity (%)±SD	ABTS scavenging activity (%)±SD	FRAP (µg TE/g±SD)	TRP (µg AE/g±SD)
RAA	90.57±0.103	96.65±0.043	400.00±0.007	287.13±0.004
RAM	91.67±0.140	96.55±0.043	429.07±0.019	292.27±0.020
RCA	90.32±0.155	96.48±0.043	337.55±0.031	271.55±0.015
RCM	91.27±0.140	96.53±0.012	378.73±0.006	283.26±0.022

RAA: Aqueous extract of *Rhododendron arboreum*; RAM: Methanolic extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*; RCM: Methanolic extract of *Rhododendron campanulatum*; SD: Standard deviation; DPPH: 1, 1-Diphenyl-2-picrylhydrazyl; ABTS: 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; FRAP: Ferric reducing antioxidant power; TRP: Total reducing power

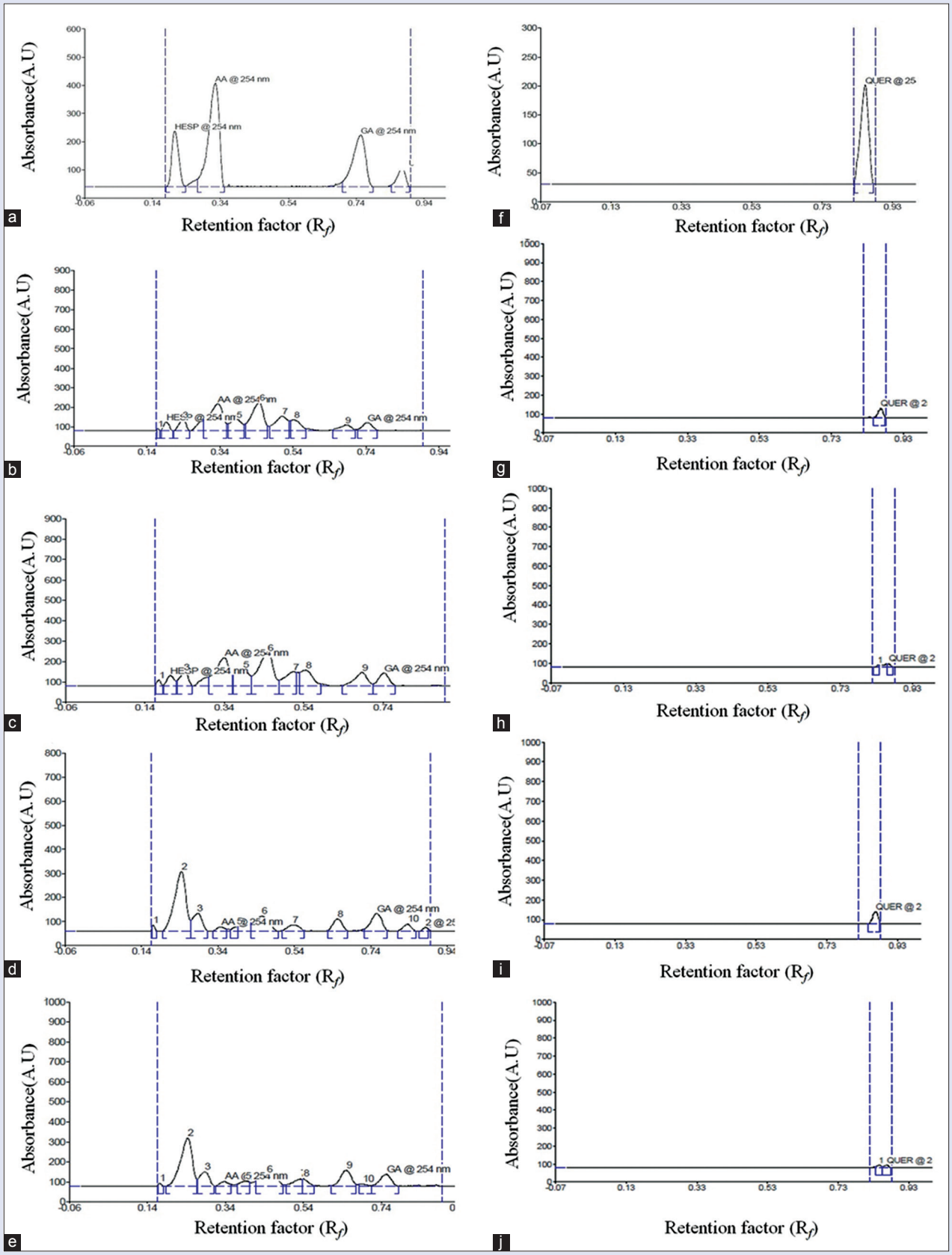

Figure 2: Densiometric chromatogram of the plant extracts. (a) Standards (HESP, GA, and AA); (b) RAA for HESP, GA, and AA; (c) RAM for HESP, GA, and AA; (d) RCA for GA and AA; (e) RCM for GA, and AA; (f) Standard (QUER); (g) RAA for QUER; (h) RAM for QUER; (i) RCA for QUER; (j) RCM for QUER. RAA: Aqueous extract of *Rhododendron arboreum*; RAM: Methanolic extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*; RCM: Methanolic extract of *Rhododendron campanulatum*; QUER: Quercetin; GA: Gallic acid; AA: Ascorbic acid; HESP: Hesperidin

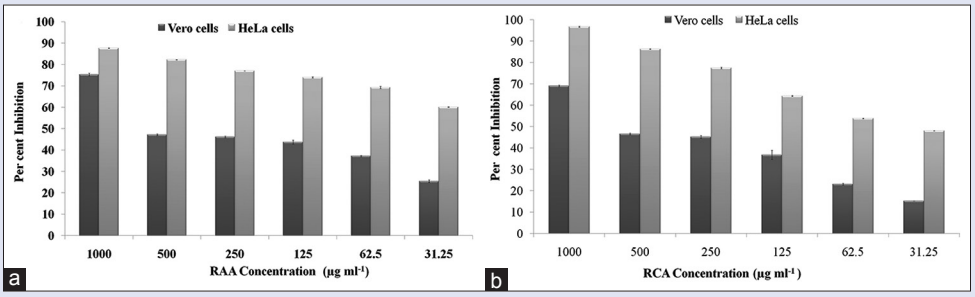

Figure 3: Antiproliferative properties of RAA (a) and RCA (b) against HeLa and Vero cell lines. RCA. RCA: Aqueous extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*

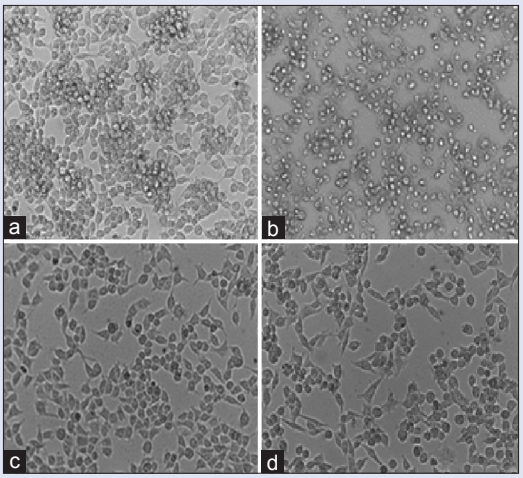

Figure 4: Effect of the extracts on the morphology of HeLa cell line after 24 h and exposure in adherent culture. (a) Untreated; (b) treated with positive control, doxorubicin; (c) treated with RAA; (d) treated with RCA. RCA: Aqueous extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*

0.24 folds, respectively [Table 5 and Figure 5]. However, RCA at the same concentration downregulated the expression of HIF-1α and VEGF to 0.47 and 0.32 folds, respectively. Doxorubicin (an anticancer drug) at a concentration of 5 µg/ml has shown almost complete inhibition of the HIF-1α and VEGF [Table 5].

Cytotoxicity in yeast model

As shown in Figure 6, the extracts were nontoxic for the growth of *S. cerevisiae* cells at the concentrations used [Figure 6]. It was observed that both the extracts did not cause any lethality to yeast cells until the concentration of 5000 µg/ml. At 10^4 µg/ml of concentration, the extracts (RAA and RCA) showed significant toxicity. H_2O_2, a known oxidant appeared to be lethal, even at a very low concentration, 42.5×10^{-4} µg/ml [Figure 6].

DISCUSSION

Rhododendron genus has enormous ethnopharmacological value.[28] Around 208 compounds have been isolated from different species of *Rhododendron* with the majority representing flavonoids and diterpenoids.[64] Flowers of *R. arboreum* have been reported to be rich in quercetin, rutin, coumaric acid, and other flavonoids.[29,33]

Similarly, *R. campanulatum* flowers and leaves are reported to have diverse range of phenolics such as gallic acid, quercetin, and campanulin, along

Table 5: Fold change in the expressions of hypoxia-inducible factor-1α and vascular endothelial cell growth factor

Treatment	HIF-1α (mean value±SD)	VEGF (mean value±SD)
Untreated control	1.0000±0.00	1.0000±0.00
Doxorubicin control	0.0015±0.00	0.0002±0.00
RAA	0.3220±0.01	0.2400±0.01
RCA	0.4700±0.11	0.32±0.06

RAA: Aqueous extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*; VEGF: Vascular endothelial growth factor; SD: Standard deviation; HIF-1α: Hypoxia-inducible factor-1

with pharmacological properties.[28,65] The PLE technique or accelerated solvent extraction was applied for extraction due to several advantages such as less time and solvent requiring process. PLE is used for the preparation of phenolic-rich extract from plant material.[66] In comparison with previous studies, the yield of the extract was found to be either higher or comparable to other studies.[42,67,68] Crude extracts were used in this study because a big lobby of herbalist believes that diverse phytochemicals in synergism have better therapeutic potential than that of alone active principles.[69,70] In addition, pure drugs are more expensive, unavailable, and unaffordable to the economically weaker population in the remote areas.

In the present study, TPC and TFC of the extracts of *R. arboreum* were found to be much higher than previous study.[38] Similarly, the RCM has shown higher TPC and TFC values when compared to other studies.[38] Higher TPC and TFC reported in the present study may be credited to the specific method of extract preparation, i.e., PLE. The extract prepared in the present study showed higher amount of quercetin and gallic acid when compared to previous studies.[29,68] The extracts showed the presence of the phenolics and flavonoids of medicinal importance, e.g., quercetin, gallic acid, hesperidin, and ascorbic acid, which are reported to have medicinal values against cancer, inflammation, and free radicals.[71-76]

All four extracts showed excellent free radical scavenging activities for DPPH and ABTS along with remarkable FRAP and TRP values. Earlier studies have shown antioxidant activities in the leaf extract of *R. arboreum*. DPPH scavenging and FRAP have been analyzed for hydromethanolic leaf extract of *R. arboreum* prepared through maceration.[38,77] *R. campanulatum,* a native of high altitude, is known for its ethnopharmacological value with very little scientific literature available on it. In a study, hydromethanolic leaf extract of *R. campanulatum* (prepared through maceration) has revealed good DPPH scavenging activity, hydroxyl scavenging activity, and ferrous ion chelating activity.[38] We reported either comparable or better free radical scavenging activity and reducing power potential, probably due to polyphenol-rich extraction through PLE. We further investigated anticancer properties of the aqueous extracts (RAA and RCA) because

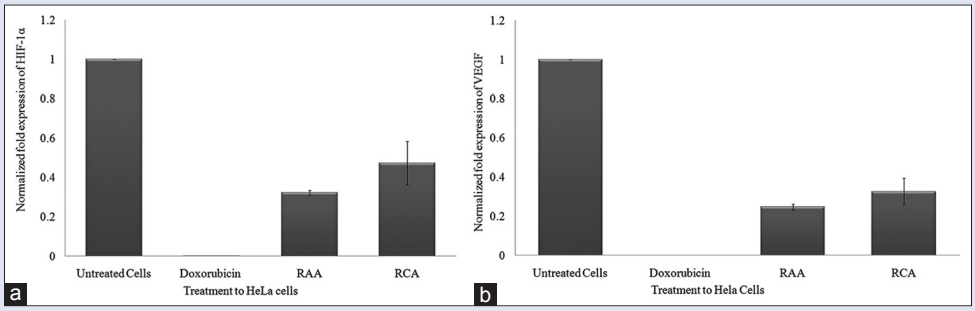

Figure 5: Effect of RAA and RCA upon HIF-1α expression (a and VEGF expression (b) in Hela cells. RCA: Aqueous extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*; VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia-inducible factor-1

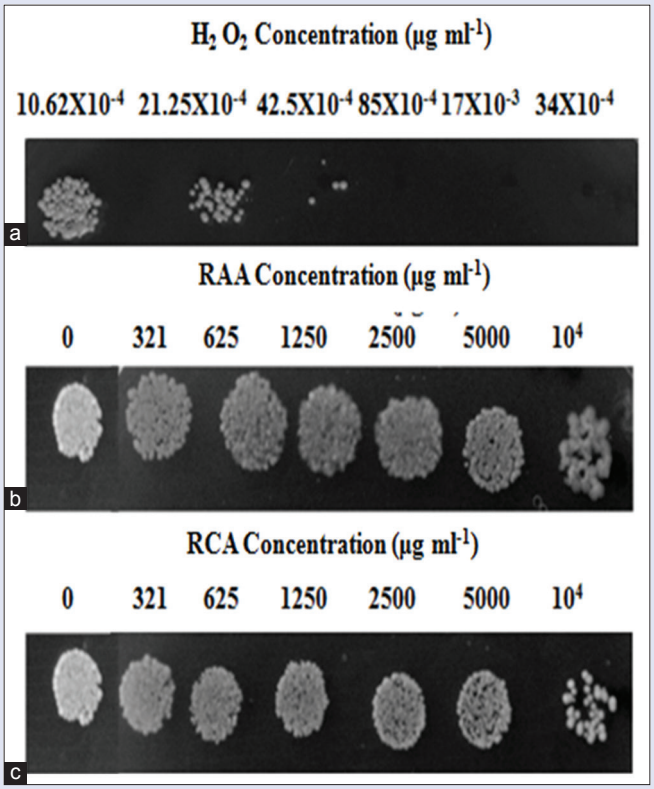

Figure 6: Effect of exposure (24 h) of the extracts on the growth of yeast cells. (a) H_2O_2 exposure; (b) RAA exposure; (c) RCA exposure. RCA: Aqueous extract of *Rhododendron arboreum*; RCA: Aqueous extract of *Rhododendron campanulatum*

aqueous extracts do not have the issue of solvent selection during formulation of the drug and are associated with low toxicity.[78,79]

The aqueous extracts of both the plants have shown approximately 50% inhibition of human cancer cell lines (HeLa) at the lowest concentration (31.25 μg/ml), but the inhibition was significantly lower for noncancerous cells (25% for RAA and 15.17% for RCA). Both the extracts have shown promising antiproliferative activity. According to the NCI, USA, if a crude extract is able to inhibit ≥50% cancer cell lines at concentration of <30–40 μg/ml, then it may be considered as antiproliferative drug.[80] In harmony to the NCI specification, RAA and RCA inhibited ≥50% cancer cells at a concentration of 31.25 μg/ml. On the contrary, both extracts showed poor inhibition of noncancerous Vero cells at a concentration of 31.2 μg/ml. The results indicated that RAA and RCA are more toxic against cancerous cell than normal cells, which is expected from an effective anticancer drug with the least side effects. In most of the cancer cells, tumor is characterized by inadequate supply of oxygen (hypoxic condition), responsible for the critical alterations in the cellular microenvironment, which results in resistance to radiation-therapy/chemotherapy and poor diagnosis.[81] HIF-1, a transcription factor, upregulates the expression of VEGF, EPO (the gene responsible for erythropoiesis), and other hypoxic genes to survive the hypoxic microenvironment of affected tissue, leading to neovascularization. Anticancer drugs are able to inhibit HIF-1 and dependent transcription of VEGF in human prostate cancer xenograft.[82] The elevation in the HIF-1α level is associated with high death rate among cancer patients.[83] Therefore, we investigated the expression of HIF-1α and VEGF mRNAs in the HeLa cells after treatment with the extracts to find out the molecular targets. RAA significantly repressed the HIF-1α (0.322 folds) and VEGF (0.24 folds). Similarly, RCA lowered the expression of HIF-1α (0.47 folds) and VEGF (0.32 folds). The antiproliferative activity of the two extracts may be due to phenol and flavonoid present in them. Phytoconstituents reported in the extracts (quercetin, hesperidin, gallic acid, and ascorbic acid) are well known to possess anticancer activities.[84,85] The extracts exposure did not affect the growth of yeast cells, even at a concentration of 5000 μg/ml. This highlights that extracts do not possess any toxicity in defined concentration against eukaryotic cells. Further investigations and toxicity studies are required to develop an effective antioxidant and anticancer therapeutics from the plants used in this study.

CONCLUSION

This study revealed the presence of phenolics and flavonoids in the leaf extracts of *R. arboreum* and *R. campanulatum*. Aqueous and methanolic extracts exhibited free radical scavenging activities and reducing potential, required to be an antioxidant. The aqueous extracts of both plants have shown promising antiproliferative activities against human cervical cancer (HeLa) cell line, whereas the extracts showed relatively lower level of cytotoxicity against normal (Vero) cell line. The RAA and RCA downregulated the mRNA levels of HIF-1α and VEGF (oncogenic factors). Both plants have potential to be developed as natural antioxidant and anticancer drug.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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