Decreased S Phase and G2/M Phase Cells by a Bioactive Fraction from the Hexane Extract of *Rhododendron aroboreum* Leading to the Apoptosis of HeLa Cells *In-Vitro*

Bibechana Timsina and Varalakshmi Nadumane[*]

School of Sciences, Department of Biotechnology, JAIN (Deemed to be University),
Near Ravindra Kalakshetra, Bangalore, Karnataka State 560027, India

(**[*]Corresponding author's e-mail: kn.varalakshmi@jainuniversity.ac.in)

Abstract

Search for novel anticancer compounds from natural sources is a priority goal of cancer biologists. *Rhododendron arboreum* is one of the most stately and impressive *Rhododendron* species. In hilly areas, the flowers of *R. arboreum* are used for the treatment of chronic diseases. The current study was aimed to evaluate the anti-proliferative property of *R. arboreum* on HeLa cells. Crude extract of the flower was prepared using serial extraction procedure with different solvents. Cytotoxicity of the extracts on HeLa, CHO and human lymphocytes were assessed using MTT assay and trypan blue dye exclusion methods. The extract was further purified by TLC and characterized by LC-MS method. The hexane extract showed significant cytotoxicity to HeLa cells. This extract was fractionated using thin layer chromatography, 6 fractions were isolated, out of which fraction 3 showed the maximum cytotoxic activity with an IC_{50} of 15 μg/mL. The mechanism of action was observed as apoptosis induction as indicated by elevated caspase activity and inhibition of cell cycle with reduced cell population in the S and G2/M phases. LC-MS analysis of this promising bioactive fraction resulted in a molecular ion fragment with an m/z value of 437.48 which is corresponding to that of brefeldin A, an anticancer compound. It can be concluded that *R. arboreum* flower has cytotoxic activity which could be due to the presence of brefeldin A, that warrants further studies on purification and characterization towards the development of highly efficient therapeutics for cancer.

Keywords: *R. aroboreum*, Anti-cancer, S phase cells, Caspase-9, Apoptosis

Introduction

Cancer is a major public health problem in developed as well as developing countries, being the 2nd leading cause for the death of humans worldwide (WHO 2008). In spite of greater advancements in the medication and therapeutic approaches to cure the disease, cancer still remains a major challenge to the scientific community with resistance and recurrence happening frequently. Search for a novel anticancer compound of natural origin has been one of the priority goals for cancer biologists. Ethnomedicinal use of plant-derived natural products play a significant role in the discovery and development of potential anticancer agents [1]. *Rhododendron arboreum* is an evergreen shrub or a small tree with a spectacular display of bright red flowers. 80 species of *Rhododendron* are found in India, with 10 sub species and 14 varieties, most of them are widely distributed in Himalayas from Kashmir to Bhutan, in the hills of Sikkim, Assam, and Manipur at altitudes of 1,500 - 5,500 m. *R. arboreum* is one of the most stately and impressive *Rhododendron* species. In hilly areas, the flowers of *R. arboreum* are used in the preparation of jams, jellies and local brew. Local people use this plant for the treatment of chronic eczema, diarrhoea, dysentery and menstrual disorders [2]. Anti-diabetic activity was also examined in *Rhododendron arboreum* flower and active compounds were isolated [3]. *Rhododendron* has long been used in the Ayurvedic system of medicine for the cure of snake bite, bites of wild animals, cancer, sores, ulcers, dysentery, menorrhagia and obstinate sciatica [4]. From the stem bark of *R. arboreum* 15-Oxoursolic acid was isolated which had considerable anticancer activity [5]. Recently, a study reported the anticancer activity of the methanol extract of *R. arboreum* leaves and flowers, but there was no study report about the anticancer mechanism of *R. arboreum* flower extract [6]. Hence, the particular study was aimed to investigate the anticancer activity and the mechanistic evaluation of *R. arboreum* flower extract on *in-vitro* cancer cell line, HeLa,

and on a normal cell line CHO. Also, an attempt was made to partially characterize the compound responsible for this bioactivity.

Materials and methods

Sample collection, authentication and preparation of extracts

The flowers of *Rhododendron* were collected from Sikkim, India. The species was identified by Dr. Shiv Kumar Sharma (Assistant Scientific Officer, Dept. of Science and Technology, Sikkim) and the herbarium voucher specimens were deposited in Jain University, Bangalore, India. The flowers were cleaned, separated and dried under the shade at the room temperature (28 ± 2 °C). The dried samples were ground into a fine powder using a dry grinder, and then kept in an air-tight container and stored in a freezer (–20 °C) before extraction. 35 g of dried powder was used for serial extraction in a soxhlet apparatus using hexane (50 mL), methanol, chloroform and water (30 mL each). The extracts were filtered and the solvent was evaporated in a rotary evaporator to obtain the dry extract. Required concentrations of the extract were prepared by dilution of the stock (1mg/mL) with sterile dimethyl sulphoxide (DMSO) [7]

Cell lines and culture

HeLa and CHO cell lines were procured from National Centre for Cell Sciences (NCCS), Pune. They were maintained in DMEM medium (HIMEDIA, India) supplemented with 10 % Fetal Bovine Serum (HIMEDIA, India). Lymphocyte isolation was performed using the blood collected from few healthy male and female individuals, about 20 years of age. The ethical guidelines for research of the Indian Council of Medical Research [8] were followed with regard to blood sampling. HiSep medium (HIMEDIA, India) was used for the isolation. Lymphocytes and CHO cells were used as control cells to assess the cytotoxicity of the extracts. The cells were incubated at 37 °C with 95 % air and 5 % CO_2. All cells were maintained below passage 20 and used in experiments during the linear phase of growth.

MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide)] assay

Hela and CHO cells growing exponentially were collected after trypsinization and plated in 96-well microtiter plates in 100 µL of culture medium and were allowed to adhere for 24 h before treatment. Increasing concentrations of hexane extract of *Rhododendron*, dissolved in DMSO, were added to different wells of the microtiter plates. Final concentration of DMSO in the culture medium was maintained at 0.4 % (v/v) to avoid solvent toxicity. The cells were incubated for 24, 48 and 72 h in the presence and absence of the extracts. Cytotoxicity was analyzed using MTT assay following the standard protocol [9]. Cytotoxicity was expressed as the concentration of the extract inhibiting cell growth by 50 %, relative to cells incubated in the presence of 0.4 % DMSO. The absorbance was read at 540 nm using the ELISA plate reader. Each experiment was performed in triplicates. The following formula was used to calculate the percent of inhibition:

Inhibition (%) = $(1-OD_s/OD)\times100$

where, OD_s = Optical density of the sample and OD = Optical density of the control.

Chromatographic separation and identification of the bioactive fraction (TLC)

To fractionate the bioactive components from *Rhododendron* flower hexane extract, it was subjected to thin layer chromatography (TLC) as per the standard methodology [10]. Chromatogram was performed using different solvent systems such as (a) Toluene: Ethylacetate: Formic acid (2.5:1:1 v/v); (b) chloroform: Acetone (6:4 & 8:4 v/v); (c) hexane:acetone (6:4 & 8:2 v/v); (d) dichloromethane: acetone (6:4 & 8:2 v/v); (e) Toluene. The chromatograms were detected with the help of a UV transilluminator (254 and 366 nm). Bioassay guided fractionation was followed for the detection of the best fraction separated in TLC. From the chromatogram developed as described above, each band was scraped, mixed with methanol and centrifuged at 3,000 rpm for 15 min. Supernatant was collected in a pre-weighed vial and kept for evaporation of the solvent and collection of the active components in dry form. The partially purified fractions obtained from preparative TLC were tested for cytotoxicity against the HeLa cells, CHO cells and the lymphocytes by MTT assay as described earlier.

LDH cytotoxicity assay

LDH Assay is a colorimetric method of assaying cellular cytotoxicity. The assay quantitatively measures a stable cytosolic enzyme lactate dehydrogenase (LDH), which is released upon cell lysis. Cells

treated with fraction 3 of *Rhododendron* for 24 h were collected by tripsinization, centrifuged at 1,000 rpm for 10 min, and 10 µL of lysis buffer was added and plated in triplicates in a 96 well plate along with the controls, positive (1 % Triton X-100) and negative (untreated). 50 µL of substrate was added to the wells and incubated in the dark for 20 min. After the incubation period, 50 µL of stop solution was added to all the wells to stop the reaction and readings were noted down at 490 nm. Percentage cytotoxicity was measured using the following formula:

$$\text{Percentage cytotoxicity} = (\text{OD}_{\text{treated}} - \text{OD}_{\text{negative control}})/\text{OD}_{\text{positive control}} \times 100$$

Caspase-9 apoptosis assay

Caspase-9 activity was assessed using the caspase-9 Colorimetric Assay Kit (G Biosciences, kit 786-205A). The assay is based on spectophotometric detection of the chromophore *p*-nitroaniline (*p*NA), after cleavage from the labeled substrate LEHD-*p*NA. The free *p*NA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-9 activity. Approximately 2×10^6 cells, treated for 24 h with *Rhododendron* fraction 3, were lysed in 500µl of lysis buffer and lysed by alternate freezing and thawing for 4 - 5 times. 50 µL of cell lysate was added to 50 µL of 2X CasPASE™ assay buffer (containing 7.5 mM DTT). Blanks were also set up by adding 50 µL of lysis buffer into the wells instead of cell lysates. 5 µL of the substrate (1mM AFC-conjugate) was added to the wells (50 mM final concentration) and the contents of the wells were mixed gently and the absorbance was detected in an Elisa plate reader at 405 nm at 0 time point (t = 0). The plates were incubated at 37 °C and the absorbance was recorded every 15 min until a significant difference in the readings occurred from those at t = 0. Percentage increase was calculated using the following formula:

$$\text{Rate of percentage increase in caspase activity over the length of the reaction time} = (\text{OD}_{\text{control/sample}} - \text{OD}_{\text{blank}})/\text{OD}_{\text{blank}} \times 100$$

All experiments were performed in triplicate and repeated at least 3 times.

Cell cycle kinetics

Cells grown in 12-well plates (5.0×10^5 cells/mL) were treated with the bioactive fraction (fraction 3) for 24 h. Briefly, cell pellets were obtained by tripsinization, washed twice with PBS and fixed overnight with 70 % ethanol at 4 °C. After incubation, cells were centrifuged again at 5,000 rpm for 10 min and washed twice with PBS. Cells were resuspended in 1 mL of PBS and in ribonuclease (100 µg/mL). Then cells were re-suspended in staining solution [50 µg/mL propidium iodide, 30 units/mL RNase, 0.1 % sodium citrate, and Triton X-100 (pH 7.8)] and incubated at 37 °C for 15 min. After incubation in the dark, fluorescence-activated cells were sorted in a FACScan flow cytometer (equipped with a 488-nm argon laser), and the data were analyzed on a MACS Quant analyser.

HPLC and Mass Spectrometric analysis of the active fraction

To further purify the active fractions, the TLC purified bioactive fraction 3 of *Rhododendron* flower was subjected to high performance liquid chromatography. The HPLC system with 2487 dual λ U-V detector (258 nm), 1525 Binary pump and ODS - C18 analytical column (4.6 I.D X 150 mm) was used with 5 µm particle size. The mobile phase consisted of solvent A: solvent B (1:1). Solvent A is 2 % acetic acid and Solvent B acetonitrile: 0.5 % acetic acid (1:1 v/v). The separation was performed using isocratic elution with a flow rate of 1.0 mL/ min. The injection volume was 20 µL and the column temperature was 25 °C. The sample and mobile phase were filtered through 0.22 µm PVDF filter before injecting them to the column. The ESI mass spectra were recorded using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). Spectra were acquired over the mass range 50 - 1,500 m/z.

Statistical analysis

All experiments were carried out in triplicates. The results were expressed as mean ± standard error values. Statistical significance was calculated using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered as statistically significant [11].

Results and discussion

Viability of HeLa cells by MTT assay

When HeLa cells were treated with the hexane extract of rhododendron flower, there was a time and dose dependent inhibitory effect on cell proliferation. Percentage viability decreased as the period of exposure to the extract increased from 24 to 48 h and then to 72 h. The extract reduced the percentage viability of HeLa cells from 79.31 % after 24 h of treatment to 45.0 % after 72 h at a concentration of 1μg/mL, while at 50 μg/mL concentration, the viability of 96 % after 24 h of treatment progressively decreased to 35 % after 72 h (**Figure 1**). This indicated the higher efficiency of the extract with increasing concentration and duration of treatment.

When CHO cells were treated with *Rhododendron* flower extract, the percentage viability of the cells at any treatment concentration and duration was higher than that observed for HeLa cells at the same tested parameters, except for 50 μg/mL concentration at 24 h treatment on HeLa cells where it was found to have a lesser effect with 98 % viability as opposed to the 70 % in case of CHO cells. At 1μg/ml of the extract treated for 24 h, the percentage viability of CHO was 95 % which reduced further to 62 % at 72 h, and at 50 μg/mL concentration the viability was 70 % at 24 h which reduced to 62 % after 72 h indicating the dose and time dependent effect of the extract, higher effect at higher dose and treatment time. **Figure 1** clearly indicates the less toxic effect of the extract to CHO cells as compared to the HeLa cells.
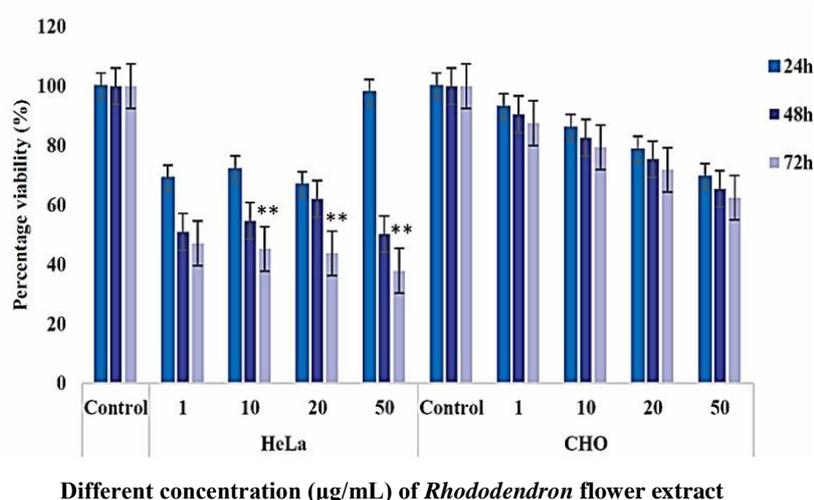

Figure 1 Cytotoxic effect of *Rhododendron arboretum* flower extract on HeLa and CHO cells.

Chromatographic separation of the bioactive compound

When thin layer chromatography (TLC) was performed using Toluene as the solvent, 6 major fractions were separated from the *Rhododendron* flower extract, which were visualized as distinct bands (**Figure 2**) with the help of a UV trans illuminator (254 and 366 nm).
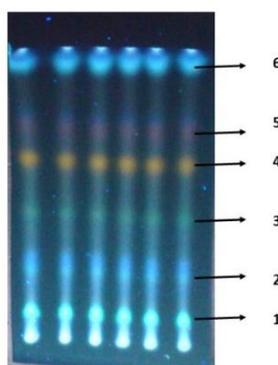

Figure 2 TLC separated fractions of *Rhododendron* flower extract.

Fractions recovered from TLC were further tested for cytotoxicity by the MTT assay. The 3rd fraction of *Rhododendron* flower extract exhibited highest cytotoxic effects than the other fractions and was chosen for further studies (results not shown). Fraction 3 at 1 μg/mL concentration, the percentage viability of HeLa cells was 90 % after 24 h and decreased to 64 % after 72 h. As the concentration was increased to 10, 20 μg/mL, the percentage viability was found to decrease in a dose dependent manner to 56 and 44 %, respectively after 72 h of treatment. At 50 μg/mL concentration the antiproliferative effect of RFF3 was very clear with 50.5, 52 and 37 % viabilities at 24, 48 and 72 h, respectively with an IC_{50} value of 15 μg/mL (**Figures 3(a)** and **3(b)**). A dose and time dependent inhibitory effect was observed even on CHO cell lines, which was comparable to that on HeLa cells. At 1 μg/mL of RFF3, the viability was 79.12 % after 24 h which reduced to 58.94 % after 72 h. At 10 and 20 μg/mL concentrations the cytotoxic effect was higher on CHO cells rather than HeLa cells. But as the treatment concentration increased to 50 μg/mL, the viability of CHO cells was found to be 52.74 % at 24 h which further reduced to 47.3 % at 72 h, while on Hela cells it was 37 % at the same concentration (50 μg/mL) and duration (72 h) of treatment. When the bioactive fraction was checked for cytotoxicity on normal human peripheral lymphocytes, a time dependent but dose independent cytotoxic effect was observed with 65 - 70 % viability at 50 μg/mL concentration, while at 1 and 10 μg/mL concentrations, a higher cytotoxic effect was seen (percentage viability ranging from 50 - 65 %) indicating RFF3 being toxic at lower concentrations to normal cells than at higher concentrations (**Figure 3(c)**).

To compare the effect of RFF3 on Hela cells to that of a cancer drug, when camptothecin (positive control) was treated on to HeLa cells at 10 μg/ml concentration for 24, 48 and 72 h, the percentage viability was 75 % at 24 h, 65 % at 48 h and 53.65 % at 72 h (**Figure 3(d)**) as compared to 85, 80 and 55 % viability of HeLa respectively by RFF3 (10 μg/mL). This indicates that the effect of RFF3 on cancer cells was comparable to that of an anticancer drug.
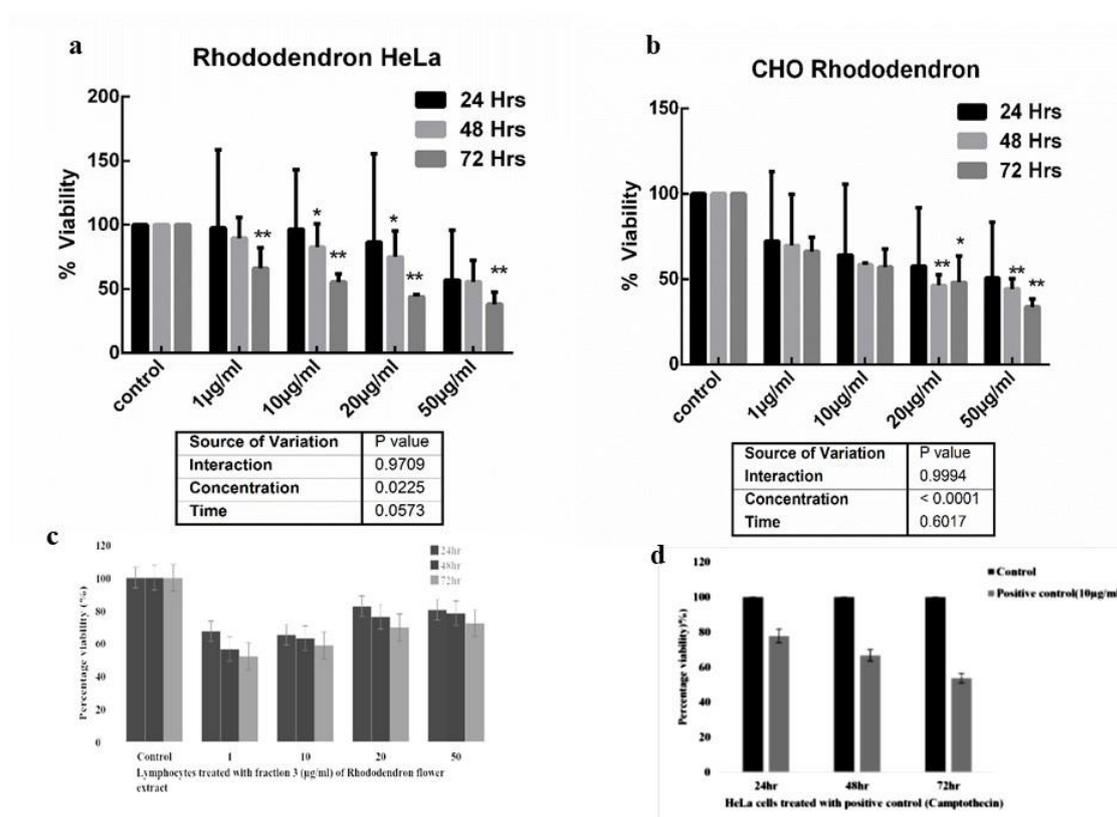

Figure 3 Cytotoxic effect of *Rhododendron* flower 3rd fraction (RFF3) to (a) HeLa; (b) CHO, (c) Lymphocytes, and (d) Effect of the positive control camptothecin (10 μg/mL) on Hela cells.

Caspase-9 activity and LDH cytotoxicity assays

Bioactive fraction 3 from *Rhododendron* flower extract increased the activity of caspase-9 in HeLa cells as well as the CHO cells, with 41 % of activity in control HeLa increasing to 85 % after treatment and in CHO cells, from 42.5 % it increased to 60 %. It is evident that the increase in activity was higher in the case of HeLa cells than the CHO cells (**Figure 4(a)**), thus confirming the apoptotic induction of cell death in the treated cells.

The cytotoxicity, as measured by LDH release by the HeLa cells treated with 50 µg/mL of fraction 3 for 24 h, was 39.63 % (**Figure 4(b)**) when compared with that of the positive control i.e., 1 % Triton-X 100, which could induce all of the HeLa cell death through cytotoxic effects. In the case of CHO cells, the treatment with RFF3 caused 19.9 % cytotoxicity. This indicates that the bioactive fraction has released higher amount of LDH when added to the HeLa cells than the CHO cells. LDH release assay is an appropriate and possibly preferable means of measuring cellular cytotoxic reactions as it directly reflects the extent of cytotoxicity of any drug or external agent effect on the cells.
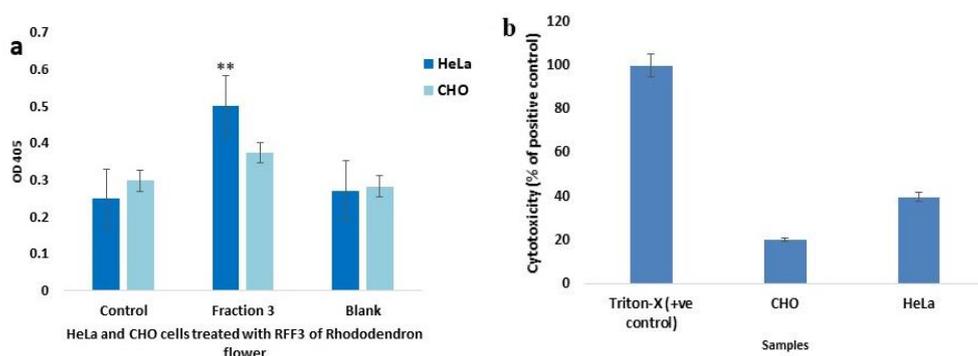

Figure 4 (a) Caspase activity of HeLa and CHO cells treated with *Rhododendron* flower fraction 3 (RFF3). (b) Cytotoxic effects of RFF3 as per LDH leakage assay.

Cell cycle analysis

When HeLa cells were treated with RFF3 fraction for 24 h, we observed decreasing cell populations in all the phases of the cell cycle as compared to the control HeLa cells. There were 24.67 % of cells in the G_0/G_1 phase as compared to 38.4 % in control cells (**Table 1**), S phase at 2.44 % as opposed to 16.58 % cells in the control and G_2/M phase having 3.03 % as compared to 17.83 % of cells in control flask. These results indicated the profound inhibitory effects of this bioactive fraction on the cervical cancer cell line as S phase reflects the synthetic phase and G2/M phase reflects the cells in the division phase of the cell cycle.

Table 1 Cell cycle stages of HeLa cells treated with RFF3 as compared to control Hela cells.

Sl. No.	Cell cycle stage	Population number	Percentage of cells (Treated)	Percentage of cells (Untreated control)
1	G0/G1	P3	24.67	38.84
2	S	P4	2.44	16.58
3	G2/M	P5	3.03	17.83

LC-MS analysis

The bioactive fraction RFF3 separated by TLC was further characterized by LC-MS analysis. As per LC-MS results, we observed 2 peaks in the chromatogram (**Figure 5(a)**), a smaller peak at retention time of 4.14 min and a larger peak at the retention time of 6.78 min. The mass spectrum of this peak showed the presence of a fragment ion with 100 % abundance with a mass to charge ratio of 437.4 (**Figure 5(b)**), with a base peak value of 453.2, which corresponds to the molecular weight of brefeldin A. brefeldin A is an anticancer compound as per earlier reports [12] and is listed in the cancer resource database (http://data-analysis.charite.de/care).
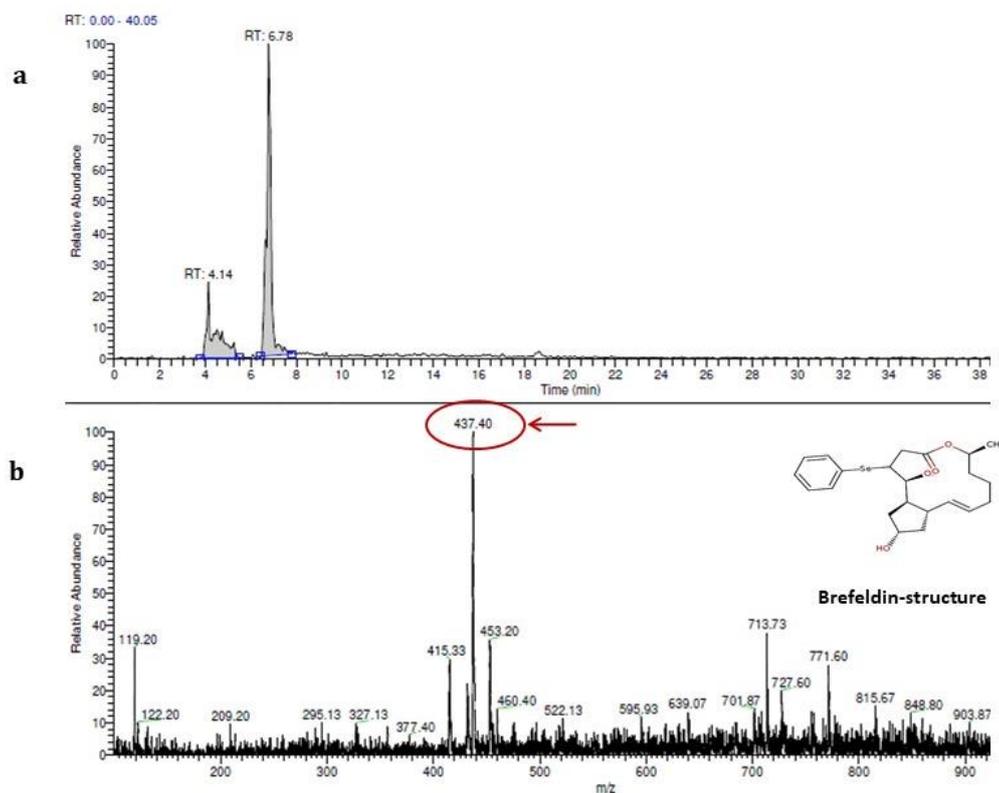
Figure 5 LC-MS analysis of fractionRFF3 from *Rhododendron* flower extract: (a) Chromatogram showing 2 peaks. (b) Mass spectrum of the peak at 6.78 min. Arrow indicates m/z value of 437.48 that corresponds to the molecular mass of the compound brefeldin A.

Cancer is a global challenge with higher number of cases and deaths reported annually around the world. Cancer is characterized by uncontrolled cell proliferation resulting from aberrant activity of various cell cycle events; therefore, cell cycle regulators are promising targets in cancer therapy. Natural compounds have provided many effective cell cycle inhibitors for cancer management, some of them already in current use [1]. *Rhododendron arboreum* is an evergreen shrub or a small tree with a spectacular display of bright red flowers, widely distributed in Himalayas from Kashmir to Bhutan. The flowers of *R. arboreum* plant are used traditionally in west Himalaya as a remedy for liver complaints and in anemia [13]. It is stated that the phytochemistry, biological activities and toxicity of *R. arboreum* are reported so far [14]. The phytochemical research on this plant led to the isolation of phenolics, triterpenoid, flavonols, flavonol glycosides and sterols. The crude extracts and isolated compounds of this plant exhibited various biological activities. They even pointed out that further studies should be carried on extracts towards the isolation of compounds from crude extracts in order to explore the full potential of this plant [14]. Though there are reports about the anticancer activity of crude extract from the leaves of this plant, the mechanism of anti-proliferative and anti-cancer activities has not been explored extensively [13]. Hence, the aim of the current study was to explore the anticancer activity, mechanism of action and to identify the active principle behind this activity from the flower extract of *R. arboreum*. Here in our study, during initial screening we found that the crude hexane extract from the flowers of this plant has promising anti-proliferative activities with IC_{50} value as low as 20 μg/mL. In a previous study, they reported aqueous extract of *R. arboreum* leaves (RAA), at 31.25 μg/mL concentration inhibited 60.12 and 25.41 % proliferation respectively of HeLa and Vero cell lines [13]. When compared to this report, the hexane extract from the flower is showing better results in our study. Following the promising results, we further tried to partially purify and fractionate the crude extract by bioassay guided fractionation through thin layer chromatography. Thin layer chromatography is a technique which separates various constituents in a mixture based upon their solubility in different organic solvents used in the mobile phase. Through this, we found the 3rd fraction (RFF3) to be having highest effect on the cancer cell line HeLa and have chosen for further studies. Inhibition of cancer cell proliferation along with inducing apoptosis are the major ways to combat cancer

progression. In our study, we could demonstrate that the bioactive fraction was indeed anti-proliferative in nature to both HeLa and CHO, with the effect being inversely proportional to time and concentration of exposure to fraction RFF3. In our results we observed highest inhibition of HeLa only at 72 h of treatment at all tested concentrations, while on CHO the effect was evident even at 24 and 48 h. But at the same tested concentrations and durations, the effect of RFF3 was comparatively lower on normal human peripheral lymphocytes, evident at higher concentrations of 20 and 50 μg/mL, proving its lesser cytotoxic effects to normal human cells.

The apoptosis inducing ability of this fraction was analyzed by caspase 9 activity measurement. in this analysis we found RFF3 had induced a 44 % increase in the caspase activity of treated HeLa cells and 17.5 % increase in CHO cells. As caspases are the main enzymes involved in inducing apoptotic cell death, these study results are indicating the anticancer mechanism of *R. arboretum* flower is through activating the apoptotic machinery causing cell death. To further analyze the anticancer mechanism, we subjected RFF3 treated HeLa cells to flow cytometry and in this study, we found a drastic reduction in S phase cells (2.44 %) and G_2/M phase cells (3.03 %) as compared to the control conditions. There were significantly fewer cells in all 3 phases as compared to the control. Due to its ability to inhibit entry of the cells to S phase, we assume that this fraction might be effective in inhibiting the proliferation of the cancer cells.

Further, the cytotoxic effects of this fraction when checked by LDH cytotoxicity assay, we could see 39.6 % cytotoxicity in HeLa and 19.9 % in CHO cells as compared to the positive control, indicating direct cytotoxicity also as another additional mechanism of bringing cell death by RFF3. The promising results prompted us to go ahead with further subjecting the bio active RFF3 to LC-MS analysis, where we observed the presence of 2 peaks in the chromatogram. The mass to charge ratio (m/z) of one of the peaks corresponds to 437.4 as per mass spectrometric results. From the database (http://data-analysis.charite.de/care/), it was found that this value corresponds to the molecular weight of brefeldin A (2,2-syn-3-[phenylseleno] brefeldin A), which was first isolated from *Penicillium decumbens* in 1958 [15]. An earlier study, reported the presence of the flavonoids, rutin (610.5) and quercetin (302.23) in *R. arboreum* extract [16]. They also reported that rutin and quercetin might be responsible for anti-cancer property of this plant. There are reports about the isolated compound from *R. arboretum* stem bark as 15-oxoursolic acid on the basis of various extensive spectroscopic techniques. 15-Oxoursolic acid revealed considerable anticancer activity with low IC_{50} values [17]. But to the best of our knowledge, this is the 1st report of brefeldin A from *R. arboreum* flower extract through our current study results. Our study results indicate the presence of compounds other than rutin and quercetin in this medicinal plant, which might be responsible for its anti-proliferative effects observed in the case of HeLa cells. Brefeldin A (BFA) was earlier reported as an important lead molecule in drug developmental studies because of its potent antifungal, antiviral and antitumor activities [18]. BFA has been isolated from soil fungi and more recently from marine fungi and has shown versatile beneficial activities [19,20]. This is not for the first time that compounds reported from microbial sources were found in plants, earlier also scientists have found similarity in structure of plant – derived compounds to that from microbial origin [21,22].

Through our current study it was demonstrated that *Rhododendron* flower has potent anticancer activity towards the cancer cell line HeLa, without significant cytotoxic effects to normal human lymphocytes and thus holds promise towards pre-clinical and clinical studies that might lead to drug discovery. More importantly, the apoptosis-inducing activity of BFA in cancer cells highlights the possibility of further developing this compound from *R. arboretum* flower as an anticancer drug molecule.

Conclusions

This study found that *R. arboreum* flower hexane extract exerted anticancer activity on human cervical cancer cells with specific manner. These were observed through changes in the cell proliferation, caspase 9 activity, LDH activity, and cell cycle stages via flow cytometry. All the results are supportive of apoptotic induction of cell death. The compounds in the LC-MS analysis of bioactive fraction RFF3 mainly yielded brefeldin A, which may be contributing as the potential compound responsible for the observed anticancer activity. Further prospective study of the potential compound (BFA) for its specificity, also effective *in-vivo* studies for validation of anticancer activity and a deeper understanding of the mechanism are essential to develop the compound into an effective anticancer drug molecule. To the best of our knowledge, the presence of brefeldin A is being reported for the first time in *Rhododendron* flowers, through our current investigations. However, further research is required to fully characterize this promising compound.

Acknowledgements

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