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ANTICANCER POTENTIAL OF RHODODENDRON ARBOREUM LEAVES CHLOROFORM EXTRACT AGAINST BREAST CANCER CELL: IDENTIFICATION OF QUERCETIN AS POTENTIAL TUBULIN ANTAGONIST VIA VIRTUAL SCREENING

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Abstract

The study was conducted to evaluate the anticancer potential of Rhododendron leaf extract and to identify the best possible lead from the reported chemical constituents of Rhododendron. Rhododendron arboreum leaves were collected and dried in a solar dryer for 7 days and were extracted with different solvents. The extracts were evaluated qualitatively and quantitatively to identify and quantify the active components. Biological evaluations such as MTT-based anti-proliferative Assay and activity against reactive oxygen species were conducted for the extracts. A tubulin assay and a cell cycle assay were performed to prove the biological activity of quercetin, which was found to have the highest affinity toward the colchicine binding site of tubulin. As per the overall result of quantitative and anti-proliferative activity, chloroform extracts portrayed excellent anti-proliferative activity (IC50 3.28 µg/ml). CRA exhibited the most potential ROS elevation in the MCF-7 cell line. Molecular docking suggested that quercetin was also found to have the highest affinity towards the colchicine binding site of tubulin (G - 12.08 Kcal/mol). The tubulin assay showed comparable inhibition of microtubule assembly by quercetin compared to paclitaxel. Further, quercetin was also found to inhibit the progression of the cell cycle beyond the G2/M phase in cell cycle analysis. These findings suggest that the CRA of rhododendron species has potential chemical constituents with anticancer potential, and they may be explored further for their effect as anticancer agents.

Index Terms: Rhododendron arboreum, Breast cancer, tubulin, anticancer, virtual screening

1) INTRODUCTION

Rhododendron arboreum, an angiospermic plant, belongs to the family Ericaceae. The name is derived from the Greek word 'rhodo', meaning rose, and 'dendron', meaning tree [1]. These plants grow at high altitudes in North-East India. More than 800 species of Rhododendron are reported worldwide; many of them have ethnopharmacological importance [2]. The juice of the flower of this plant R. arboreum is consumed as an

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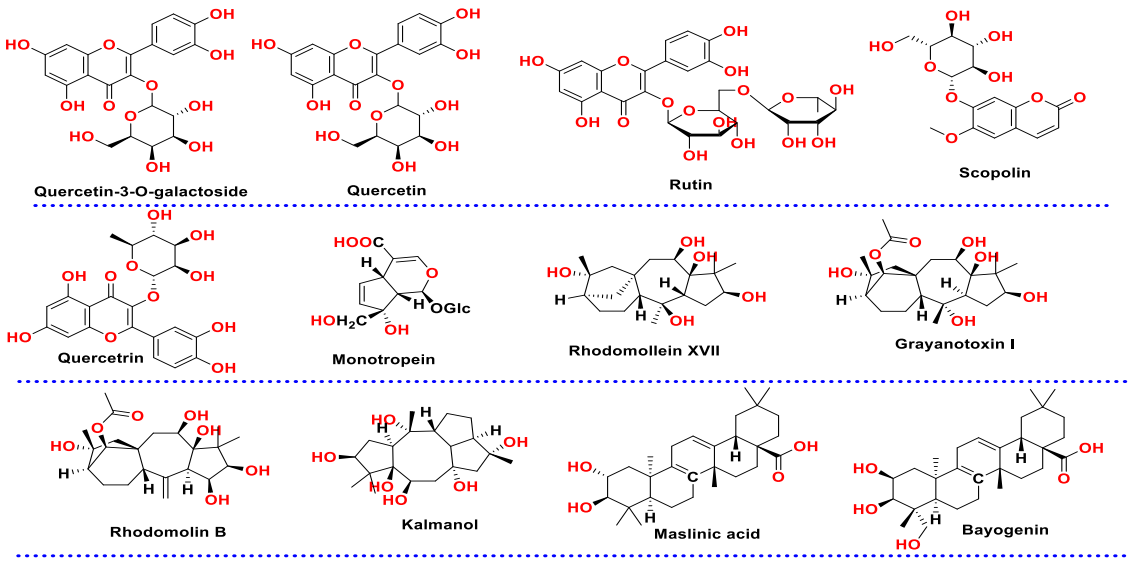
adaptogenic seasonal drink in Uttarakhand due to the presence of several phytoconstituents such as epicatechin, quercetin, syringic acid, rutin, quercetin, and coumaric acid [3]. R. arboreum is reported to possess medicinal and pharmacological properties such as hepatoprotective, antioxidant, immunomodulatory, anti-inflammatory, anti-diabetic, and antinociceptive etc., [4]. This plant consists of several phytoconstituents, including quercetin, protocatechuic acid, chlorogenic acid, gallic acid, epicatechin, and oleanane triterpene [5]. Several studies have investigated the cytotoxic effects of different species of the genus Rhododendron. Park and Kim et al., (2008) demonstrated that the essential oil fraction obtained from Rhododendron mucronatum exhibits a cytotoxic effect on human immortal keratinocyte (HaCaT) cancer cells. Acetone extract from Rhododendron ponticum leaves was cytotoxic on human prostate cancer (PC-3) cell lines [6]. Demir et al. also showed that Rhododendron luteum flower extract has a selective cytotoxic effect against human colon and liver cancer cells [7]. Sajid Ali and colleagues isolated 15-oxoursolic acid from R. arboretum and discovered significant anticancer activity with IC50 values of 2.3 0.1 mol, 4.9 0.2 mol, 9.2 0.2 mol, and 10.3 0.1 mol against MDR 2780AD (human ovarian carcinoma), Hep G2, H157 (squamous cell carcinoma), and NCI-H226 (non-small cell lung), respectively. In contrast, A498 activity was Vandana et al., investigated the methanolic extract of the Rhododendron arboreum leaves and flowers for antioxidant and anticancer potential. They found that these extracts have significant potential for growth inhibition of cancer cells [8], [9]. Furthermore, numerous chemical constituents are reported from the genus Rhododendron with immense bioactivity [10]. Key chemical constituents are portrayed in Fig. 1.

Fig 1: Key chemical constituents reported in Rhododendron arboreum



Keeping in view the immense biological attributes of Rhododendron, in the current research, we explored the anticancer potential of leaves-derived extract. The work was further extended to identify the best possible lead from the reported chemical constituents

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of Rhododendron via virtual screening as a tubulin inhibitor. Conclusive results further suggested quercetin possessed potent anticancer potential with a tubulin inhibitory profile comparable to colchicine.

2) MATERIAL AND METHODS

2.1 Materials

The solvents (petroleum ether, ethyl acetate, and methanol) required for extraction were purchased from Avra, S.D. Fine chemicals, India, were used without any further purification. Spots were visualized under UV light, and an iodine chamber and an aluminum chloride solution were used as a derivatizing agents. For weighing purposes, a Sartorius analytical balance (BSA224S-CW) was used. For drying, a tray dryer (NSW India) was used. Glassware for Soxhlet assembly was purchased from JSGW. The solvent concentration was done using an ILMVAC rotary evaporator. Quercetin was purchased from Sigma Aldrich, India, and was used without further purification.

2.2 Plant material procurement and processing

Approximately 5 kg of leaves of R. arboreum were collected from Pinani village, Pauri Gahrwal, Uttarakhand, India. The collection site was precisely located at 30.1976 °N, 76.5215 °E. Leaves were sundried for 7 days until the moisture was removed. Furthermore, the leaves were dried using a tray drier (Lab India) at 40 ˚C for 5 more days. The leaves were completely dried and turned brown by this time and were ground coarsely using a kitchen grinder, and the powder was weighed and packed. We obtained 1.9 kg of dried powder, which was carried forward for extraction and other biological activities.

2.3 Extraction

We used an accelerated solvent extractor (Speed Extractor: Buchi) for extraction. In brief, out of 1.9 kg of dried powder, we weighed (citizen weighing balance) 140.67 g finely powdered (prestige grinder) for extraction use. After mounting the device, extracts were prepared with the help of a speed extractor using different solvents (per 500 ml) ranging from polar to non-polar (petroleum ether chloroform, ethyl acetate, and methanol). The respective extracts were dried using a rotary evaporator into greasy form and finally powdered and dried using a lyophilizer system.

2.4 Qualitative analysis of selected phytochemicals

Flavonoid’s determination

1 mg of dried powder extracts was heated with 10 ml of ethyl acetate in boiling water for 3 min. The mixture was filtered separately, and the filtrates were diluted with 1 mL of ammonia solution. There was the formation of layers that were allowed to separate by letting the tubes be in a resting state for 10 minutes, and observing the colour [11-14]. Terpenoids determination

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Different extracts were used to test the presence of terpenoids by mixing the extracts in 2 ml of chloroform. Then, concentrated H2SO4 was added carefully, forming a layer, and colour observation was done [11], [12], [13], 14].

Steroids determination

Acetic anhydride (2 ml) and H2SO4 (2 ml) were added to extracts and observed for color changes [11], [12], [13], 14].

Tannins' determination

Extracts were boiled with 5 ml of 45% ethanol for 5 minutes. Each extract was filtered and diluted with distilled water, and two drops of ferric chloride were added [11], [12], [13], 14].

Saponins Assay

A small quantity of extracts was diluted with 4 ml of distilled water. The mixture was shaken continuously and then observed while standing for a regular break [11], [12], [13], 14].

2.5 Quantitative analysis of phytochemicals

2.5.1 Estimation of proteins (Bradford Assay)

Different quantities of extracts (1µl, 2 µl and 3 µl) were taken in a 96-well plate and dissolved in PBS, followed by a 20 µl addition of Bradford reagent. Mixed well on a shaker for a few minutes, read at 595 nm on a plate reader, and then compared with a standard curve (BSA taken as standard) to determine the concentration of proteins in the samples [15].

2.5.2 Estimation of phenolic compounds

Different quantities of extracts (0.5 µl and 5 µl) were dissolved in 80% methanol and then incubated for 5 min, followed by adding 50 µl of Folin-Ciocalteau reagent (2 N). Dark incubation was given for 1 h, and then the absorbance was read at 765 nm spectrophotometrically and compared with the standard curve for determining phenols in the samples. Gallic acid (10 mg/ml) was standard [16].

2.5.3 Estimation of reducing sugars

Estimation of reducing sugars was done by using the dinitrosalicylic acid (DNSA) method. Small quantities of extracts were taken, dissolved in 1 ml of DNSA, boiled for 10 min, and cooled at room temperature. Read at 560 nm on a spectrophotometer and compared with the standard curve of reducing sugars (Glucose was used as standard (10 mg/10 ml) [17].

2.5.4 Estimation of starch

Extracts were mixed with 3 ml of distilled water and 4 ml of 52% perchloric acid. The mixture was kept in an ice bath for 20 minutes. Then 3 ml of anthrone reagent was added to the mixture. The sample was boiled at 100 °C for 10 minutes before being cooled to

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room temperature. The samples were read at 630 nm spectrophotometrically and compared with a standard glucose curve to determine the concentration of starch in the sample [18].

2.6 Biological investigation

2.6.1 In vitro cell line studies

Cell culturing and maintenance

Breast cancer (MCF-7) and normal cells (MCF-10A) used in assays were-procured from NCCS Pune, India. All the biological grade chemicals were from Sigma Aldrich and Hi-media India. Cells were grown in culture flasks using Dulbecco's Modified Eagle Medium (DMEM) media, supplemented with 10% foetal bovine serum (FBS), 1X penicillin-streptomycin antibiotic solution, and then incubated at 37 ˚C in a humidified atmosphere containing 5% CO2. Sub-culturing of cells was done when the cancer cell lines had attained 70-80% growth. All the sterile conditions were maintained during the culturing and handling of cells used herein [19].

2.6.2 MTT-based anti-proliferative Assay

Approximately 8000 cells (counted using Trypan Blue dye) were seeded in each well of 96 well plates. Cells were incubated and serum-starved for synchronisation before treatment with investigational compounds. After the specified incubation time, media was discarded, and cells were washed using 1X PBS. Thereafter, MTT dye dissolved in PBS (5 mg/ml) was added to each well (10 µl) and further incubated in a CO2 incubator for 3 - 4 h. Formazan crystals thus formed were dissolved in biological grade Dimethyl sulfoxide (DMSO) and absorbance was read on a microplate reader at 570 nm. The results were performed in triplicate and are represented as mean ± SD. Tamoxifen and Erlotinib were taken as positive controls for result validation [20].

2.6.3 Preparation of stock and working solution of extracts

The extracts were dried and stock solutions were prepared by diluting them in biological grade DMSO with the highest stock of 10 mg/ml, which was further sonicated to ensure the complete solubility of the crude materials. The stock solution was further serially diluted to prepare working solutions of 25, 5, and 1 µg/ml using biological grade DMSO, and it was ensured that the highest concentration of DMSO did not exceed 0.5% in cell culture.

2.6.4 Detection of reactive oxygen species

We analysed selected extracts and compounds for their effect on ROS levels using H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) dye. The fluorescence intensity of the samples was measured using a BD C6 flow cytometer. Cells were grown in 35 mm dishes and maintained under standard conditions. Investigational compounds were incubated for 24 h with cancer cells before treatment. All the procedures were performed according to reported protocols. [21].

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2.6.5 Ligand selection via virtual screening and its corroboration

The reported chemicals (Fig. 1) were selected (downloaded or drawn using ChemBiodraw® Ultra Software) from Rhododendron arboretum, and colchicine as a reported inhibitor of tubulin protein. The chemical structures were converted into .sdf format and were further refined using LigPrep.[22]. The X-ray three-dimensional crystal structure of tubulin (PDB ID: 1SAO) [23] was retrieved from the Protein Data Bank. The protein consisted of 896 amino acid residues (α-subunit 451 amino acids; β-subunit: 445 amino acids) and existed as a heterodimer. The protein was further refined for missing sidechains and loops using the protein preparation wizard of Schrodinger software (release 2020-1), finally followed by optimization and minimization [24]. The grid was drawn around the colchicine binding site, molecular docking was performed using Glide (XP-Module), and outputs were represented as the G score [25].

2.6.6 Tubulin Assay

The fractionation of soluble and polymerized tubulin was done using MCF-7 cells as per the process defined by Legault and colleagues [26]. After drug treatment, around 5 X 106 cells in 100 mm petri-dishes were washed with PBS at 37 ˚C and harvested in 1 ml of PBS containing 0.4 µg/ml of paclitaxel. Cells were further centrifuged and lysed in microtubule-stabilizing buffer and further centrifuged at 12,000 x g for 10 min at 4 ˚C. The supernatants containing soluble tubulin were mixed with 2X Laemmli sample buffer. Pellets containing the polymerized tubulin were resuspended in 250 µl of water, followed by two thawing cycles, and finally resuspended in Laemmli sample buffer. Samples were analysed by western blot using an anti-α-tubulin antibody. Furthermore, the polymerization of bovine tubulin was measured according to Beyer et al. [27]. Bovine tubulin (1.8 mg/ml; Sigma) was added to ice-cold polymerization buffer (PEM: 80 mmol PIPES, 0.5 mmol EGTA, 2 mmol MgCl2, 10% glycerol, and 1 mmol GTP) and centrifuged at top speed in a microcentrifuge for 5 minutes at 4 °C. The supernatant (100 µl/well) was immediately added to a 96-well plate, which contained 10 µmol FZ or dimethyl sulfoxide control in PEM buffer. After adding tubulin, we placed the plate in the spectrophotometer maintained at 37 °C. The absorbance was measured at a time interval of 5 minutes for 2.5 hours at 340 nm.

2.6.7 Cell cycle analysis of quercetin against breast cancer cells

Cell cycle analysis was done to identify progression in cell cycle after treatment with investigational compounds. Propidium Iodide (Sigma Aldrich) was used as a dye and results were analysed using a BD Accuri C6 flow cytometer [28].

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3) RESULT AND DISCUSSION

3.1 Extraction

Extracts of R. arboretum were prepared in petroleum ether, ethyl acetate, chloroform, and methanol using an accelerated solvent extractor. Various extracts and their dried weights are provided in Table 1.

Table 1: Different solvent extracts and abbreviated codes

Sr. No.	Extracts	Abbreviated Codes	Dried extract weight (g)
1.	Petroleum ether extract	PERA	17.29
2.	Ethyl acetate extract	EARA	31.56
3.	Chloroform extract	CRA	33.45
4.	Methanol extract	MORA	39.73

3.2 Qualitative analysis of selected phytochemicals

The extracts from plants showed the presence of flavonoids, terpenoids, tannins, steroids, and di-terpenes in their extracts as defined in Table 2.

Table 2: Qualitative analysis of phytochemicals

Qualitative analysis of phytochemicals					
Extracts	**Flavone**	**Terpenes**	**Steroid**	**Tannin**	**Saponins**
PERA				+	+
EARA	+			+	
CRA		+		+	+
MORA	+		+		

3.3 Quantitative analysis of phytochemicals

3.3.1 Estimation of proteins (Bradford Assay)

Further, the extracts were also analyzed for the presence of proteins. EARA showed a high concentration of proteins with respect to other extracts in 1 ml, i.e.88 mg, and the lowest showed by PERA, i.e., 0.07 mg.

3.3.2 Estimation of phenolic compounds

Considering the phenolic content, MORA has shown the highest concentration of phenols at about 10.89 mg/ml and CRA has shown the lowest concentration, which is 0.583 mg/ml. Based on these results, it was seen that MORA is rich in phenolic compounds.

3.3.3 Estimation of reducing sugars

Lastly, we also analyzed extracts for the presence of reducing sugars. The results showed CRA contains the highest concentration of phenols at about 361.9 mg/ml and EARA shows the lowest concentration at 54.8 mg/ml. On the other hand, PERA and MORA showed equal quantities of reducing sugar, which is 199.7 mg/ml. Based on these results,

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the CRA extract was rich in reducing sugars (quantitative estimation is compiled in Table 3).

3.3.4 Estimation of starch

Considering the quantification of starch, CRA showed the highest concentration of starch, i.e., 164.792 mg/ml, while EARA showed the lowest concentration near about 3.6254 mg/ml. PERA showed a 43.8 mg/ml concentration, and MORA showed about 40.002 mg/ml. Based on the results, it is seen that CRA is rich in starch content.

Table 3: Estimation of Protein, Phenolic, Starch and Reducing sugar concentration in various extracts of Rhododendron leaves

Extracts	Protein concentration (mg/ml)	Phenolic concentration (mg/ml)	Starch concentration (mg/ml)	Reducing Sugars concentration (mg/ml)
PERA	0.07	2.453	43.865	199.7
CRA	3.8	0.583	164.792	361.9
EARA	88	3.454	36.254	54.8
MORA	1.8	10.89	40.02	199.7

3.4 Biological investigation

3.4.1 In vitro cell line studies

3.4.2 MTT-based anti-proliferative Assay

Different extracts of Rhododendron leaves were screened for their cytotoxic activity against the MCF-7 cancer cell lines. Anticancer activity of the leaves extract was evaluated by measuring cancer cell viability. Cell viability was measured by treating the cells with different extracts at different concentrations (1, 5, 25 µg/ml) in MTT assay. PERA has weak activity at lower concentrations, but it has a 50% inhibitory effect on cancer cell lines at 25 g/ml. Among all the other extracts, CRA was the most potent extract that showed maximum inhibition at 5 and 25 µg/ml. The results suggested that CRA portrayed excellent anti-proliferative activity followed by the EARA, PERA and MORA in MCF-7 cell line (Fig. 2A and 2B). Positive controls of the assay had an IC50 of 2.5 µmol (Erlotinib) and 2.1 µmol (Tamoxifen). IC50 values of extracts are compiled in Table 4.

Considering the results, extracts were analyzed for their selectivity using normal breast cells MCF-10A. The results suggested that the extracts did not induce any significant toxicity up to the highest concentration employed, suggesting the selective anticancer potential of the extracts (fig.2).

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Fig 2: Percent viability (A) and inhibition (B) of MCF-7 cell lines after treatment with various extracts of Rhododendron leaves at different concentrations of 1, 5, 25 µg/ml.

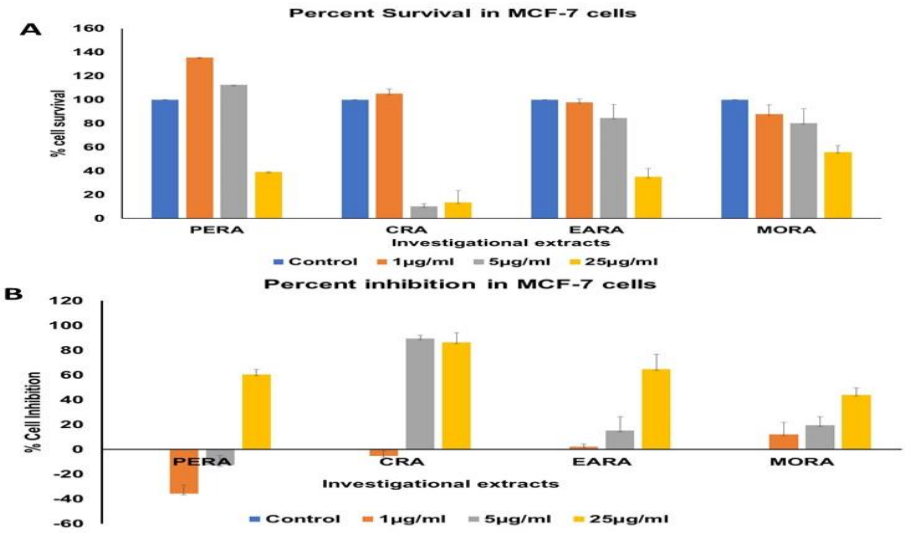
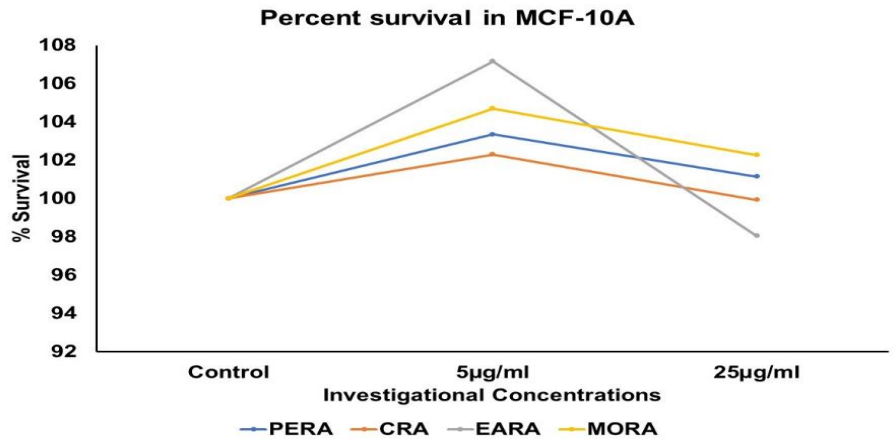


Table 4: Half maximal inhibitory concentration of various extracts of Rhododendron leaves at different concentrations of 1, 5, 25 µg/ml on MCF-7 Cells

Sr.no.	Extracts	IC_{50} (µg/ml)
1.	Petroleum ether extract	21.86
2.	Ethyl acetate extract	19.16
3.	Chloroform extract	3.28
4.	Methanol extract	> 25

Fig 3: Percent viability of MCF-10A cell lines after treatment with various extracts of Rhododendron leaves at different concentrations of 5 and 25 µg/ml.



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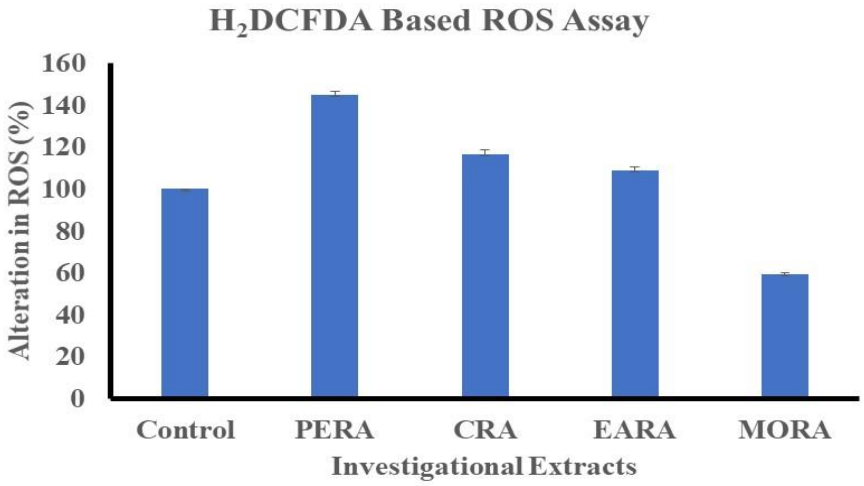
3.4.3 Detection of reactive oxygen species

ROS analysis using H_2DCFDA dye suggested the extracts were able to decrease the ROS inside the MCF-7 cells. The ROS upsurge was directly correlated with the anticancer potential of individual extracts. CRA suggested the most potential ROS elevation. The results are significant since cancer cells have a significantly higher ROS threshold than normal cells. So, the elevation of ROS beyond the threshold eventually affects the cell death pathway, and cancer cells undergo cell death via apoptosis or necrosis.

Fig 4: The bar diagram depicts the percent change in ROS after 24 hours of incubation with extract on MCF-7 cells. The Assay was performed using H2DCFDA dye.



3.4.4 Ligand selection via virtual screening and its corroboration

The virtual screening enabled molecular docking suggested that quercetin exhibited potential affinity towards the colchicine binding site of tubulin. The quercetin showed a binding affinity of G: -12.08 Kcal/mol. G values of Rutin and Quercetin-3-O-galactoside were 11.94 and 11.95 Kcal/mol, respectively (Table 5). The quercetin was found to perfectly occupy the colchicine binding site inside tubulin (Fig. 5). The important interactions portrayed by quercetin were H-Bonding by SER97, AGN112, SER110, ARG248, ASP218, and THR217. The p-p stacking was observed between the aromatic ring of quercetin and HIS216 and PHE245. Based on the results, quercetin with the highest affinity to the colchicine binding site of tubulin was used for further studies.

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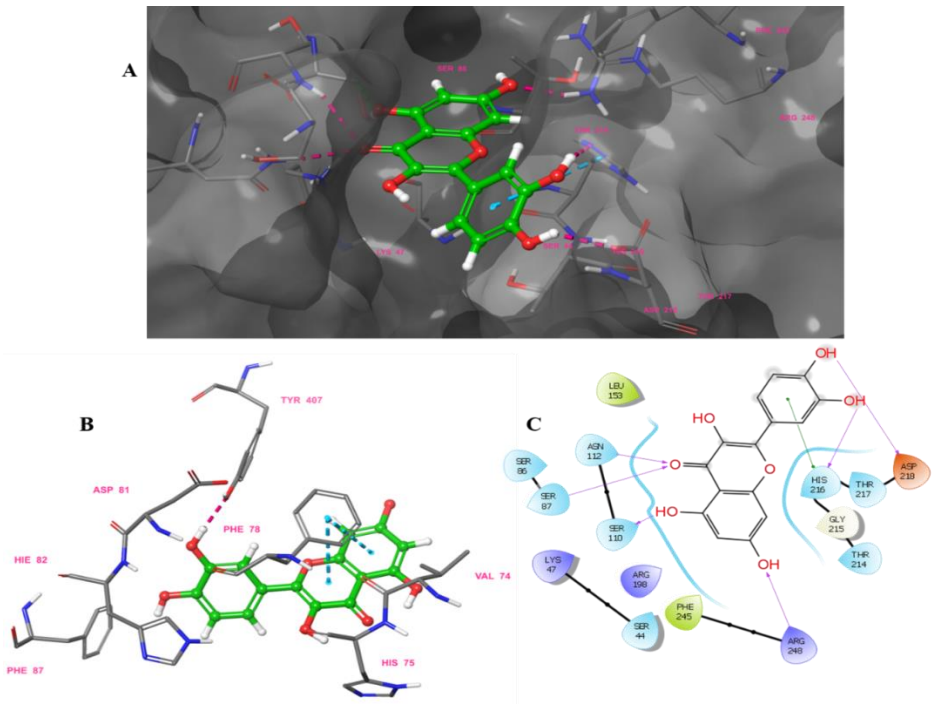
Table 5: Docking scores of key chemical constituents present in Rhododendron species

S No	Compounds Name	Docking Score (Kcal/mol)
1	Quercetin	-12.08
2	Rutin	-11.947
3	Quercetin -3-O-galactoside	-11.455
4	Scopolin	-10.524
5	Grayanotoxin I	-9.953
6	Monotropein	-9.931
7	Maslinicacid.sdf	-9.923
8	Bayogenin.sdf	-9.881
9	Rhodomolin B	-9.653
10	Rhodomollein XVII	-9.653
11	Kalmanol	-9.419

Fig 5: Illustration of binding affinity of quercetin within colchicine binding site of tubulin. A. Depicts surface image of quercetin at binding site; B. depicts the 3D pose of quercetin with important interacting amino acids within the active site; C. resembles the 2D pose of quercetin with important interacting amino acids at colchicine binding site



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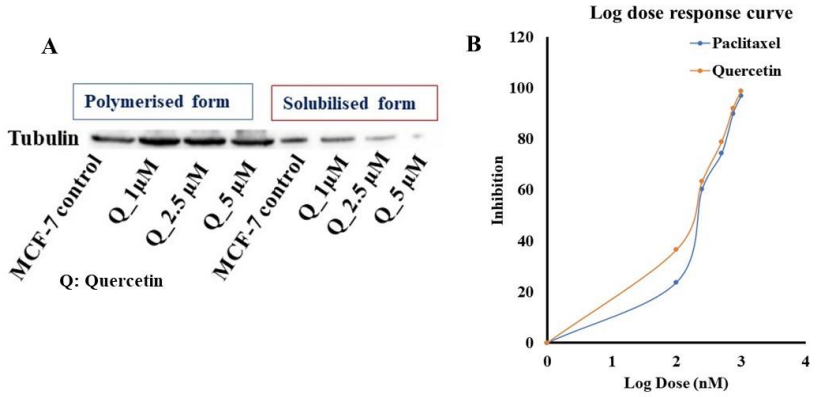
3.4.5 Tubulin assay

Tubulin is one of the highly-conserved components of eukaryotic cells, providing a structural framework for the cells and assisting in their division. Biologically, tubulin is a heterodimer of α and β subunits of microtubules with a molecular mass of ~110 kDa. The GTP allows the microtubules to polymerize to form tubulin or depolymerize into microtubules themselves. In vivo and in vitro, in the presence of gtp, tubulin heterodimers can polymerize (assemble) to form microtubules.The tubulin assay suggested quercetin was able to inhibit the assembly of microtubules by interfering with GTP. The Assay was performed at a broader concentration (100, 250, 500, 750, and 1000 nmol) of quercetin and paclitaxel was kept as a positive control. The analyses suggested a comparable inhibition of microtubule assembly by quercetin (IC_{50}: 212.96 nmol) and paclitaxel (IC_{50}: 223.76 nmol), suggesting the potential of quercetin as a tubulin inhibitor (Fig. 6B). Quercetin was also found to affect polymerization and de-polymerization of microtubules as deduced by measuring the tubulin content (polymerized and depolymerized form) in MCF-7 cells (Fig. 6A). Quercetin also exhibited an IC_{50} of 1.34 µmol towards MCF-7 cells as deduced by the MTT assay.

Fig 6: A. Blot represents the effect of quercetin on tubulin polymerization and de-polymerization. MCF-7 cells were employed and labelled with a β-tubulin antibody; B. The log dose-response curve represents the inhibitory effect of quercetin on microtubule-based polymerization performed using a pure enzyme.



3.4.6 Cell cycle analysis of quercetin against breast cancer cells

To analyse the effect of quercetin on cell cycle progression, we performed a propidium iodide-based assay. The quercetin was found to inhibit the progression of the cell cycle (Fig. 7) beyond G2/M phase, a peculiar inhibitory pattern of reported tubulin inhibitors.

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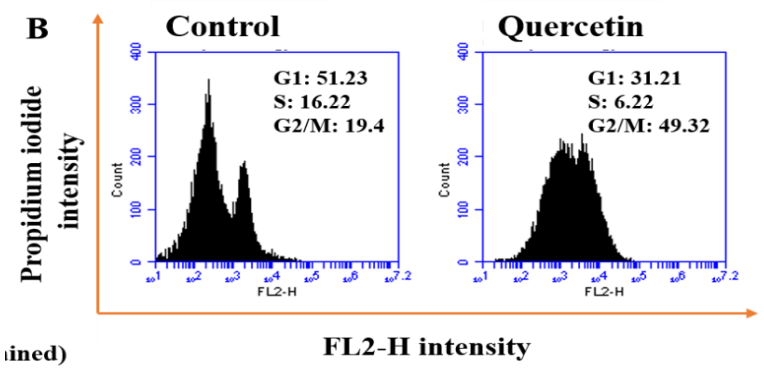
Fig 7: Halt in cell cycle progression as analysed by propidium iodide-based Assay



4) CONCLUSION

In a nutshell, we successfully explored the anticancer potential of Rhododendron arboretum native to Uttarakhand, India. The fine powder of dried leaves was subjected to extraction using petroleum ether chloroform, ethyl acetate, and methanol. The qualitative analysis of extracts further revealed the presence of flavonoids, terpenoids, tannins, steroids, and di-terpenes. Chloroform extract further suggested the highest concentration of phenols presence. The anticancer evaluation of extracts revealed that chloroform extract showcases potent anti-proliferative potential among the tested extract. Extracts on normal cells revealed their selective anticancer potential towards breast cancer cells. To add new dimensions to the current work, we shortlisted key chemical constituents in Rhododendron species and attempted to explore their anticancer potential via tubulin inhibition. Virtual screening enabled molecular modelling led us to uncover quercetin as one of the top-scoring (G score) among all compounds analysed via in silico technique. The corroboration of in silico results with in-vitro based biological assays deciphers the anticancer potential of quercetin via tubulin inhibition with a comparable profile as of paclitaxel, a reported tubulin inhibitor. The quercetin was also found to exhibit potent anticancer potential towards MCF-7 cells and was able to alter the ROS levels significantly. Quercetin halts the progression of the cell cycle beyond the G2/M phase. These findings suggested that chloroform extracts from rhododendron species have potential chemical constituents with anticancer potential and may be explored further for their effect as anticancer agents. Results also suggested that in-silico-guided exploration of major chemical constituents from Rhododendron species allowed us to identify quercetin as a microtubule-interfering agent and could serve as a potential antimitotic and anticancer lead.

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Author contribution

Gunjan Tiwari: Data analysis and interpretation, drafting the article, Critical revision of the article

Vikesh Kumar Shukla: Conception or design of the work, Final approval of the version to be published

Conflicts of interest

The authors have no conflicts of interest

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