SPECIMEN FORMAT FOR THESES OF MONTH

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Sub Subject Heading:	:	---
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Title of the thesis	:	Exploring the Anti-Cancer Potential of *Rhododendron arboreum* Sm. in Gastric Cancer using *In Vitro* and *In Vivo* Models.
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Abstract within 300 words:

Rhododendron arboreum Sm. an evergreen species of the Ericaceae family, has been traditionally used in medicine for various ailments. This study investigated its phytochemical composition, antioxidant capacity and anticancer potential against gastric cancer. Qualitative and quantitative phytochemical screening of leaf and flower extracts was performed using standard protocols, while functional groups were confirmed through FT-IR, NMR and GC-MS. Antioxidant activity was assessed by DPPH, ABTS, H_2O_2, LPO and FRAP assays. Cytotoxicity against AGS gastric cancer cells was evaluated using the MTT assay, while *in vivo* efficacy was tested in a C57BL/6 mice model with histopathological and biochemical analysis. *In silico* approaches including SwissADME, network pharmacology and molecular docking were applied to evaluate pharmacokinetics, protein interactions and binding stability of bioactive compounds. Methanol extracts of both leaves and flowers demonstrated higher phytochemical content compared to other solvents. Antioxidant assays revealed stronger radical scavenging activity in leaf extracts (DPPH IC_{50}: 56.8 µg/mL; ABTS IC_{50} 60.7 µg/mL) relative to flowers, though flowers exhibited superior reducing power in the FRAP assay. Methanol extracts also displayed potent cytotoxicity against AGS cells and significantly suppressed tumor growth in mice. GC-MS analysis identified several bioactive compounds, with network pharmacology linking ten of them to 113 proteins, including 22 associated with gastric cancer. Functional enrichment highlighted pathways such as EGFR tyrosine kinase inhibitor resistance. Among these, quercetin demonstrated the strongest binding affinity, with molecular dynamics confirming stable interactions over 100 ns. In conclusion, *Rhododendron arboreum* exhibits significant phytochemical richness, antioxidant activity and anticancer potential. The identification of quercetin as a lead compound, together with *in vivo* tumor suppression, underscores its therapeutic promise against gastric cancer and provides a foundation for further pharmacological exploration.

i) Major objectives:

1. To evaluate the qualitative and quantitative phytochemical analysis of *R. arboreum* leaf and flower extracts.
2. To determine the antioxidant potentials and anti-proliferative effects of *R. arboreum* extracts on gastric cancer cells.
3. To validate the efficacy of *R. arboreum* leaf and flower extracts using gastric cancer induced mice model (C57BL/6).

4. To decipher the prominent targets associated with gastric cancer through network pharmacology and elucidate the molecular interaction of bioactive compounds with the identified targets.

ii) Hypothesis:

The proposed study was framed to analyse the following hypothesis

- *Null Hypothesis*: The *R. arboreum* methanol leaf and flower extracts do not possess strong anti-gastric cancer activity.
- *Alternative Hypothesis*: The *R. arboreum* methanol leaf and flower extracts possess strong anti-gastric cancer activity.

iii) Methodology:

Medicinal plants have always been an essential part of human health and offer natural remedies for a variety of ailments. They contain bioactive compounds such as alkaloids and flavonoids that have therapeutic properties. In all cultures, these plants are valued for their traditional healing practices and offer treatments for ailments ranging from common cold to chronic diseases. The present research aims to discover new applications and optimize their potential benefits, highlighting their continued importance to medicine and wellbeing.

The leaves and flowers of *Rhododendron arboreum* were collected from Pedung village in West Kameng district of Arunachal Pradesh, India and authenticated at BSI (Botanical Survey of India) Coimbatore. Tamil Nadu. (No.: BSI/SRC/5/23/2021/Tech on 25/02/2021)

The collected leaves and flowers were completely washed and dried in the shade for a fortnight. The dried samples were powdered separately and stored in an airtight container for further analysis. 20 g of the powdered leaves and flowers were soaked in 200 ml of aqueous, methanol, ethanol, acetone, chloroform and petroleum ether in a separate conical flask and incubated for 72 hours at room temperature and then in incubator shaker at 150 rpm, then filtered and further concentrated by rotary vacuum evaporator at 125 rpm in water bath (~48^{o}C).

PHASE I

1. Leaf and Flower extracts were tested for the presence of bioactive compounds using standard methods (Pandey and Tripathi, 2014; Dhawan and Gupta, 2017).
2. Quantitative analysis is done by the following standard methods

- Estimation of flavonoids (Eom *et al*., 2016) using 10% aluminium chloride solution and potassium acetate solution.
- Estimation of total carbohydrates (Hedge and Hofreiter 1962) using the anthrone reagent.
- Determination of proteins: Lowry method (Waterborg, 2009) using alkaline copper solution and Folin-Ciocalteu reagent.
- Determination of total tannins: Folin-Ciocalteu method (Folin and Ciocalteu, 1927) using sodium carbonate solution.
- Determination of total alkaloids: Bromocresol green method (Shamsa *et al.,* 2007) involving the reaction with bromocresol green reagent.
- Determination of total phenolics (Chedea and Pop, 2019), using the Folin-Ciocalteu reagent and sodium carbonate.

3. FTIR- Fourier Transform Infrared Spectroscopy (Lorenz, 2020) employing 100 mg KBr pellets as encapsulation in sample discs, which scanned the spectral range from 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.
4. NMR- Nuclear Magnetic Resonance (Huang *et al.*, 2014) using Bruker AVANCE III instrument operating at 600.13 MHz and 25°C, ^{1}H NMR spectra were recorded with 16/64 scans, an acquisition time of 6.50 min per spectrum point, and a 5 s relaxation delay.
5. GC-MS-Gas Chromatography-Mass Spectrometry (Tayade *et al.,* 2022). Agilent GC-MS (CH-GCMSMS02) used with 8890 GC and 7000 GC/TQ. For interpretation of Gas Chromatography-Mass Spectrometry (GC-MS) peaks were matched with the National Institute of Standards and Technology (NIST) database.

PHASE II

1. Radical scavenging activity of *R. arboreum* Sm. methanolic extracts of leaf and flower were evaluated for the following assays.
    - DPPH Radical Scavenging assay (Blois, 1958).
    - ABTS radical scavenging assay (Re *et al.,* 1999).
    - H_2O_2- Hydrogen Peroxide Assay (Halliwell and Gutteridge 1985).
    - Lipid Peroxidation Assay (Repetto *et al.*, 2012).
    - FRAP Assay- Ferric reducing Antioxidant Power Assay (Benzie and Strain, 1996).
2. Cytotoxicity study using AGS cell lines- The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay by Cardile *et al.*, 2005 method.

3. For detecting cell death by flow cytometry, cells were stained with FITC Annexin V to identify early apoptosis and co-stained with propidium iodide (PI) to differentiate late apoptosis and necrosis.
4. Toxicity study- Brine Shrimp Lethality Assay (Hamidi *et al.,* 2014) was conducted to screen the toxicity of plant extracts by exposing *Artemia salina* to different concentrations for 24 hours and calculating the LC_{50} based on mortality rates.
5. Statistical analyses- The analyses were performed in triplicate to ensure accuracy, and the results were presented as mean values accompanied by their standard deviations. Subsequently, IC_{50} values for the antioxidant assays were calculated using the linear regression method. The experimental data underwent analysis using one-way factorial ANOVA, followed by the Tukey multiple range test at $\alpha = 0.05$.

PHASE III

1. *In vivo* studies on Docetaxel-Induced experimental mice model

Using the C57BL/6 model for *in vivo* cancer studies allows to observe treatment effects in living organisms, offering insights into tumor growth and therapy efficacy that cannot be gained from cell cultures alone. Therefore, the present study was conducted to evaluate the following parameters.

- Enumeration of Red Blood Cells (Raabe *et al.,* 2011).
- Differential Leukocyte Count (Nivedhita *et al.,* 2020).
- Estimation of Hemoglobin: Sahli's acid Haematin Method (Thakkar *et al.,* 2021).
- Estimation of Total Cholesterol (Jain *et al.,* 2020).
- Estimation of Triglycerides (Bertolo and Bogusz, 2022).
- High-Density Lipoprotein Cholesterol (Cole, 1997).
- Determination of Total Proteins (Waterborg, 2009).
- Determination of superoxide dismutase (SOD) (Kakkar *et al.,* 1984).
- Determination of Catalase (CAT) (Sinha *et al.,* 2022).
- Determination of Glutathione Peroxidase (GPx) (Rotruck *et al*., 1973).
- Determination of reduced glutathione (GSH) (Ellman,1959).
- Determination of lipid peroxidation (LPO) (Ohkawa *et al*., 1979).
- Myeloperoxidase Activity (MPO) (Wei and Frenkel, 1991).
- Histological Examinations (Ahmed *et al.,* 2015).

2. Statistical analysis- Values are expressed as mean ± SEM. Statistical significance (p) was calculated by one-way ANOVA followed by Dunnett's test (n=6); ns - non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, calculated by comparing treated groups with the control group.

PHASE IV

1. *In-silico* Studies- SWISS ADME to predict the pharmacokinetics, drug-likeness and potential ADME (absorption, distribution, metabolism and excretion) properties of small molecules in drug discovery.
2. Network Pharmacology to understand the interactions between selected drugs, targets and biological networks, helping to reveal multi-target mechanisms and predict therapeutic effects and potential side effects.
3. Molecular Docking to predict the interaction between a small molecule (ligand) and a target protein, to evaluate the binding affinity and stability of potential drug candidates.
4. Molecular Dynamic Simulation (MDS) to model the physical movements of atoms and molecules over time, allowing the study of molecular interactions, stability and conformational changes in biological systems.

iv) Findings:

PHASE I

Preliminary qualitative analysis revealed the presence of both primary and secondary metabolites in the aqueous, methanol, ethanol, acetone, chloroform and petroleum ether extracts of *R. arboreum* leaves and flowers.

In the quantitative analysis of *R. arboreum* leaves and flowers using methanol, ethanol and acetone as extraction solvents where leaf extracts showed flavonoid content of 117.1 mg/g, followed by alkaloids (75.95 mg/g), tannins (47.84 mg/g) and phenols (40.62 mg/g), whereas, flower extracts of flavonoids reaching the highest level (139.6 mg/g), followed by tannins (80.01 mg/g), phenols (47.16 mg/g) and alkaloids (32.31 mg/g). The results showed that methanol extracts had the highest concentrations of phytochemicals, confirming its effectiveness in extracting a wide range of polar compounds and facilitating additional investigations into the plants bioactive compounds for cancer prevention and treatment.

FTIR spectroscopic analysis of *R. arboreum* leaves and flowers extract revealed a diverse array of functional groups, indicating the presence of various bioactive compounds. The analysis highlighted significant functional groups related to phenols, amines, hydrocarbons, ketones and alkenes in both extracts. This confirms the rich chemical composition of *R. arboreum*, contributing to its potential therapeutic properties.

NMR analysis of *R. arboreum* leaves and flowers extract revealed various proton environments, indicating a complex molecular structure. The presence of deshielded protons suggests vinylic or aromatic groups, while peaks associated with protons adjacent to electronegative atoms or carbonyls indicate functional groups like ethers or alcohols. A significant presence of aliphatic protons was also noted, suggesting long-chain aliphatic compounds. The observed patterns imply the molecule contains multiple methyl and methylene groups, highlighting its potential complexity.

GC-MS analysis of the crude extract from *R. arboreum* leaves and flowers identified a total of 135 compounds in the leaves and 184 in the flowers, excluding derivatives and concentrating on specific interests. Distinct peaks for each component were detected through chromatography, with each peak corresponding to a specific compound. The identification of these compounds was accomplished by analyzing the retention time and mass spectra linked to the peaks.

PHASE II

The present study evaluated the radical scavenging activity of leaves and flowers extract of *R. arboreum* using the DPPH assay. DPPH showed that the IC_{50} values for *R. arboreum* leaves and flowers extract were 56 µg/mL and 67 µg/mL, respectively. These results indicate potent radical scavenging activity for both extracts.

The methanol extract of *R. arboreum* leaves and flowers exhibited an IC_{50} value of 60 µg/mL and 65 µg/mL in the ABTS assay. These results indicated variability in radical scavenging activity between the leaves and flowers.

In the hydrogen peroxide scavenging assay, the IC_{50} values for the methanol leaves extract were found to be 53 µg/mL, while the flowers extract showed an IC_{50} value of 57 µg/mL. These results indicated that both extracts possess significant antioxidant potential against hydrogen peroxide-induced oxidative stress.

In the lipid peroxidation inhibition (LPO) assay, the IC_{50} values for methanol extracts from the leaves and flowers of *R. arboreum* were 56.6 µg/mL and 60.4 µg/mL respectively, demonstrating significantly higher inhibition compared to ascorbic acid alone ($p < 0.01$) tested in egg yolk. The antioxidant efficacy of *R. arboreum* extracts emphasizes the potential therapeutic value in mitigating oxidative damage related to lipid peroxidation.

The Ferric Reducing Antioxidant Power (FRAP) assay for *R. arboreum* flowers and leaves extract revealed a clear trend in antioxidant activity across varying concentrations (0 to 250 µg/mL). Both the extracts showed an increasing FRAP values with higher concentrations, indicating enhanced reducing power.

The crude extracts of *R. arboreum* flowers and leaves were dissolved in saline water to prepare a 1mg/mL stock solution. Different volumes (100, 250, 500, 1000, 1500 µL) of the extracts were added to saline solution for the brine shrimp lethality bioassay. Thirty shrimps were introduced to each concentration, with brine solution as a blank and potassium dichromate (1mg/mL) as a positive control. Shrimp mortality was monitored over 24 hours, and both the extracts showed significantly lower toxicity compared to potassium dichromate, with only 1 to 13 mortalities at the highest concentrations.

The cytotoxic activity of *R. arboreum* leaves and flowers methanol extract. showed the strong cytotoxicity, with inhibition ranging from 43.5% at 125 µg/mL to 66.2% at 1000 µg/mL for leaves, while the flowers extract exhibited inhibition between 46.7% and 59.9% at the same concentrations. The IC_{50} values were 0.541 mg/mL for flowers extract and 0.841 mg/mL for leaves extract. Flow cytometry analysis further confirmed a higher rate of apoptosis in cells treated with flowers extract when compared with leaves extract. Both extracts induced apoptosis in a concentration-dependent manner, highlighting their cytotoxic potential.

PHASE III

In this study, forty C57BL/6 mice (25–30 g) from Biogen, Bangalore, were housed under controlled conditions. The experiment, approved by IAEC (Ethical Approval No. AIW: IAEC.2023:11), involved seven groups. Group I was the control and Group II received AGS cells. Groups III to VII received AGS cells and treatments (standard drug and plant extracts) at varying doses. After 8 weeks, tumor analysis was conducted, including stomach homogenization and centrifugation. The supernatant was collected for *in vivo* studies. Initial body weights averaged 20.6±1.86 g in Group I, with changes observed across treatment groups. Stomach weight varied with treatments.

Tumor development was observed at a 200 mg/kg dose combined with AGS induction. *R. arboreum* methanol flowers and leaves extract were tested for anti-hematologic neoplasm effects. Groups II, VI, and VII showed increased RBC counts, while WBC and lymphocyte counts remained similar. Hemoglobin levels increased in Groups VI and VII. Serum markers (urea, uric acid, creatinine) indicated kidney stress.

Antioxidative effects were observed, especially at 200 mg/kg, reducing tumor incidence and increasing body weight. Lipid peroxidation was elevated in Group VI. Antioxidant enzyme activities (SOD, CAT, GPx, GSH) were lower in cancer-induced mice but improved with *R. arboreum* leaves high dose treatment ($P < 0.001$). Myeloperoxidase (MPO) activity was higher in Group II (untreated) compared to all other treatment groups.

Histological analysis of the Group I (control) revealed a normal gastroesophageal junction with consistent non-neoplastic gastric foveolar and squamous epithelium, showing no abnormalities across the stomach layers. In contrast, Group II (untreated) exhibited lesions lined by low-grade neoplastic columnar epithelium with gastric glands containing paneth and goblet cells, while Group III (docetaxel) displayed a non-neoplastic gastric columnar lining epithelium with edematous submucosa. In Groups IV (low dose) and V (high dose) *R. arboreum* flower extracts, the microscopic analysis revealed a gastroesophageal junction with non-neoplastic gastric columnar lining epithelium. Groups VI (low dose) and VII (high dose) *R. arboreum* leaf extracts showed non-neoplastic gastric foveolar epithelium folded and lined by columnar epithelium, indicating a complete response to treatment.

The flower extract-treated groups demonstrated limited immune cell infiltration in the tumor microenvironment, with scattered lymphocytes, macrophages and neutrophils, suggesting a subdued immune response and limited immunomodulatory capacity. On the other hand, leaf extract treatment showed a complete response with well-organized foveolar and mucosal cells, indicating restoration of normal gastric epithelium and highlighting its potent therapeutic potential in reversing tumor-associated damage. Both the extracts demonstrated cytoprotective effects, such as reduced ulcer areas, decreased edema and leukocyte infiltration, particularly at a 200 mg/kg body weight dosage combined with AGS induction.

PHASE IV

Ten key chemical compounds from the leaves and flowers of *R. arboreum* were identified, including beta-sitosterol and quercetin. These compounds were mapped to their

respective human gene targets, with 958 gastric cancer-related targets identified. A comparison revealed 115 unique targets, with 22 common targets selected for further study.

Enrichment analysis identified key pathways, including EGFR tyrosine kinase inhibitor resistance, and highlighted significant genes like EGFR, ESR1 and MMP9. The molecular docking study assessed the binding affinities of five compounds beta-sitosterol, quercetin, gamma-sitosterol, alpha-amyrin, and 2,3,4,6-tetramethoxystyrene against four key target proteins (PDB IDs: 1B3D, 1ERR, 6GQO, and 6S9B). Quercetin exhibited the highest binding affinities, with energies of -8.68 kcal/mol for 1B3D, -6.407 kcal/mol for 6S9B, -6.053 kcal/mol for 6GQO and -5.374 kcal/mol for 1ERR.

The docking conformations of critical regulatory targets ESR1, KDR, MMP9, and EGFR identified through KEGG analysis were evaluated. These results suggested quercetin as a promising candidate for further investigation due to its strong interactions with above mentioned proteins. The present study conducted molecular docking of ligands and standard drug-docetaxel with gastric cancer-related proteins (MMP9, EGFR, KDR, and ESR1) to assess binding affinities, which are critical for tumor invasion and progression. The results were compared with bioactive ligands of *R. arboreum* extracts to evaluate the therapeutic potential and mechanisms of both docetaxel and the natural compounds in inhibiting gastric cancer pathways.

The molecular docking results indicated that quercetin exhibited the highest binding affinity against MMP9 (PDB ID: 1B3D) with a score of -8.68 kcal/mol, surpassing docetaxel's score of -5.318 kcal/mol. This suggested quercetin may offer better inhibition potential for MMP9. Other ligands, such as gamma-sitosterol (-4.53 kcal/mol), beta-sitosterol (-4.36 kcal/mol), and 2,3,4,6-tetramethoxystyrene (-4.23 kcal/mol), exhibited moderate affinities, while alpha-amyrin had the weakest affinity (-3.56 kcal/mol), still comparable to docetaxel.

For EGFR (PDB ID: 6S9B), quercetin again showed the strongest binding affinity at -6.40 kcal/mol, outperforming docetaxel (-3.783 kcal/mol). Other ligands, including 2,3,4,6-tetramethoxystyrene (-3.29 kcal/mol), beta-sitosterol (-2.67 kcal/mol), and gamma-sitosterol (-1.31 kcal/mol), displayed weaker interactions, with alpha-amyrin showing the lowest score (-1.16 kcal/mol). In KDR protein (PDB ID: 6GQO), quercetin maintained the score of -6.05 kcal/mol, whereas docetaxel showed binding affinities of -6.621 kcal/mol. Other ligands had lower affinities: gamma-sitosterol (-3.05 kcal/mol), beta-sitosterol (-3.04 kcal/mol) and alpha-amyrin (-3.01 kcal/mol).

Lastly, for ESR1 (PDB ID: 1ERR), quercetin showed the highest binding affinity at -5.37 kcal/mol, slightly lower than docetaxel (-5.698 kcal/mol). Other ligands, including 2,3,4,6-tetramethoxystyrene (-3.28 kcal/mol), beta-sitosterol (-3.05 kcal/mol), and alpha-amyrin (-2.62 kcal/mol), exhibited moderate binding, while gamma-sitosterol had the lowest affinity (-2.52 kcal/mol).

Molecular dynamics simulations (MDS) were performed over approximately 100 nanoseconds to assess the stability of protein-ligand complexes under varying physiological conditions. The Root Mean Square Deviation (RMSD) analysis indicated distinct fluctuations and stabilization patterns across different protein-ligand complexes. For complex 1B3D, the first fluctuation occurred at 13.10 ns, with protein and ligand RMSD values of 2.47 Å and 1.53 Å, respectively. Complex 6S9B exhibited fluctuations starting at 4.00 ns, reaching higher RMSD values (protein: 2.77 Å, ligand: 4.14 Å) before stabilizing at 14.10 ns. The 6GQO complex showed a significant initial ligand RMSD of 13.32 Å at 4.30 ns, which was stabilized in 5.54 Å at 40.50 ns. In the case of 1ERR, fluctuations began at 3.40 ns, with subsequent stabilization and later fluctuations at 63.80 ns.

Root Mean Square Fluctuations (RMSF) analysis revealed varying degrees of flexibility in both protein chains, indicating dynamic structural changes. The ligand properties of quercetin, including radius of gyration, RMSD, intramolecular hydrogen bonds, molecular surface area, solvent accessible surface area and polar surface area, remained stable throughout the simulations, suggesting quercetin's suitability for drug discovery. Overall, the simulations confirmed quercetin's superior binding energy compared to other compounds, underscoring its potential role in modulating gastric cancer pathogenesis through key target proteins.

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