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Original Research Article

Antitumor activity of NRF2 modulating fraction of anacyclus pyrethrum using EAC solid tumor model

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Abstract

Background: Breast cancer remains a leading cause of mortality among women worldwide, with increasing resistance to chemotherapy and radiotherapy. Overexpression of Nuclear Factor Erythroid 2-Related Factor 2 (NRF2) and its downstream effectors, such as NQO1 and HO-1, contributes to cancer progression and therapy resistance. The present study explores the antitumor potential of the NRF2-modulating fraction of *Anacyclus pyrethrum* (AP) using an EAC (Ehrlich Ascites Carcinoma) solid tumor model.

Objective: To evaluate the in vitro and in vivo efficacy of *Anacyclus pyrethrum* ethanolic extract (EEAP) in inhibiting tumor progression via NRF2 pathway modulation.

Methods: Ethanolic extract of *Anacyclus pyrethrum* was prepared using Soxhlet extraction. In vitro cytotoxicity was assessed using SRB assays on breast cancer cell lines, and in vivo antitumor activity was evaluated in EAC-induced Swiss albino mice. Tumor volume reduction, histological changes, and NRF2-associated signaling markers were assessed.

Results: The ethanolic extract of *Anacyclus pyrethrum* demonstrated significant in vitro cytotoxicity, with maximum inhibition observed at a concentration of 31.25 µg/mL after 72 hours. In vivo, tumor volume reduction and enhanced necrosis were observed in treated groups. Histopathological analysis showed substantial cell death in tumor tissues of mice treated with EEAP. NRF2 signaling inhibition, confirmed by NQO1 assays, revealed a dose-dependent response to EEAP, suggesting downregulation of NRF2-mediated pathways.

Conclusion: This study highlights the potential of *Anacyclus pyrethrum* as a rich source of phytochemicals with significant antitumor properties. The extract effectively inhibits NRF2-mediated tumor progression, supporting its future development as a therapeutic agent for breast cancer management.

Keywords: Breast cancer, *Anacyclus pyrethrum*, NRF2 modulation, EAC tumor model, Anticancer phytochemicals

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1. Introduction

Cancer remains one of the leading causes of mortality worldwide, accounting for approximately 10 million deaths annually, with breast cancer being a predominant contributor among women.[1] In India, breast cancer accounts for an alarming incidence rate of 25.8 cases per 100,000 women and a mortality rate of 12.7 per 100,000.[2] The resistance of cancer cells to standard therapies such as chemotherapy and radiotherapy, often mediated by dysregulated signaling pathways, underscores the need for novel therapeutic approaches.[3]

The Nuclear Factor Erythroid 2-Related Factor 2 (NRF2) pathway plays a critical dual role in cancer biology. While NRF2 activation protects normal cells by enhancing antioxidant responses, its overexpression in cancer cells promotes tumor growth, resistance to chemotherapeutic agents, and metastasis.[4] Elevated NRF2 levels upregulate downstream effectors, such as NQO1 and HO-1, which contribute to therapy resistance and poor prognosis in breast cancer patients.[5]

Natural products derived from medicinal plants have garnered attention as potential sources of anticancer agents

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due to their efficacy, affordability, and lower toxicity.[6] *Anacyclus pyrethrum*, a medicinal plant traditionally used for its immunostimulatory and antioxidant properties, contains phytochemicals that modulate various molecular pathways.[7] Previous studies have highlighted the pharmacological benefits of *A. pyrethrum*, but its potential as an anticancer agent, particularly through NRF2 pathway modulation, remains underexplored.[8-9]

This study investigates the antitumor potential of an ethanolic extract of *Anacyclus pyrethrum* (EEAP) in vitro and in vivo using an Ehrlich Ascites Carcinoma (EAC) solid tumor model. The objectives include assessing the efficacy of the extract in modulating the NRF2 pathway and its downstream targets to induce tumor inhibition.

2. Materials and Methods

2.1. Plant collection and extract preparation

Anacyclus pyrethrum (L), also called African pyrethrum, tigendesste, igendess, and akarkarha, is a plant indigenous to Algeria, Spain and Morroco. The roots of A. pyrethrum are advised for treating a variety of conditions, including paralysis of the tongue and limbs as well as toothaches, angina, salivary secretion, digestive issues, lethargy, and female infertility. They are used to prevent diseases and cure conditions including gout and sciatica in the form of cream-based animal fats. *Anacyclus pyrethrum* root samples were collected from a local farm in Mysuru, Karnataka, and authenticated by Professor Siddaramiah, Department of Horticulture, Government of Karnataka. The roots were shade-dried and ground into a fine powder. Soxhlet extraction was performed using 500 g of powdered material and ethanol as the solvent. The extract was concentrated to yield a sticky ethanolic extract (10.5 g, 9% yield), which was stored at 4°C for further analysis.

3. In Vitro Cytotoxicity Assessment

The cytotoxic potential of the ethanolic extract of *Anacyclus pyrethrum* (EEAP) was evaluated using the Sulforhodamine B (SRB) assay on breast cancer cell lines.

1. **Cell culture:** BT474 breast cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% L-glutamax.
2. **SRB assay:** Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. EEAP at concentrations of 100, 250, and 500 µg/mL was added, and cells were incubated for 48 h. After fixation with trichloroacetic acid, cells were stained with 0.4% SRB, washed, and solubilized with Tris buffer. Absorbance was measured at 540 nm using a plate reader.

Cytotoxicity was expressed as the percentage of cell growth inhibition relative to the untreated control, calculated using the formula:

Cell growth (%) = (Average absorbance of the test/Average absorbance of the control) X 100.

4. In Vivo Antitumor Studies

The antitumor efficacy of EEAP was evaluated in Swiss albino mice bearing Ehrlich Ascites Carcinoma (EAC) solid tumors.

Animal Grouping and Treatment: Thirty-six mice (4–6 weeks old, 26–30 g) were divided into six groups (n = 6 each):

1. Group: Normal control, received 0.5% sodium CMC.
2. Group: EAC control, received EAC cells and 0.5% sodium CMC.
3. Group: Positive control, treated with cisplatin (3.5 mg/kg i.p.).
4. Groups 4–6: Treated with EEAP at 2.5, 5, and 10 mg/kg i.p., respectively.

Tumors were induced by injecting 5×10^6 viable EAC cells into the right thigh. Treatments began on Day 12 post-induction and continued every alternate day until Day 24.

1. **Tumor volume and survival analysis:** Tumor volume was measured using Vernier calipers every five days, and mean survival time (MST) and percentage increase in lifespan (%ILS) were calculated.

2. **Histopathological examination:** Tumors were fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H&E) for histopathological analysis.

4.1. NQO1 assay

NAD (P) H: Quinone oxidoreductase 1 (NQO1) activity was measured to evaluate NRF2 pathway modulation. Tumor cells were lysed, and the protein content was quantified. Cell lysates were incubated with NQO1 cocktail (± dicumarol) and absorbance was recorded at 610 nm every minute for 30 minutes. Enzyme activity was calculated using standard formulas.

4.2. Statistical analysis

Data were expressed as mean ± SD and analyzed using one-way ANOVA. A p-value < 0.01 was considered statistically significant.

5. Results

5.1. Extraction yield

The Soxhlet extraction of *Anacyclus pyrethrum* roots yielded 10.5 g of ethanolic extract from 500 g of dried root powder,

corresponding to an extraction efficiency of 9%. **Figure 1** shows the flowchart of sample extraction using Soxhlet extraction.

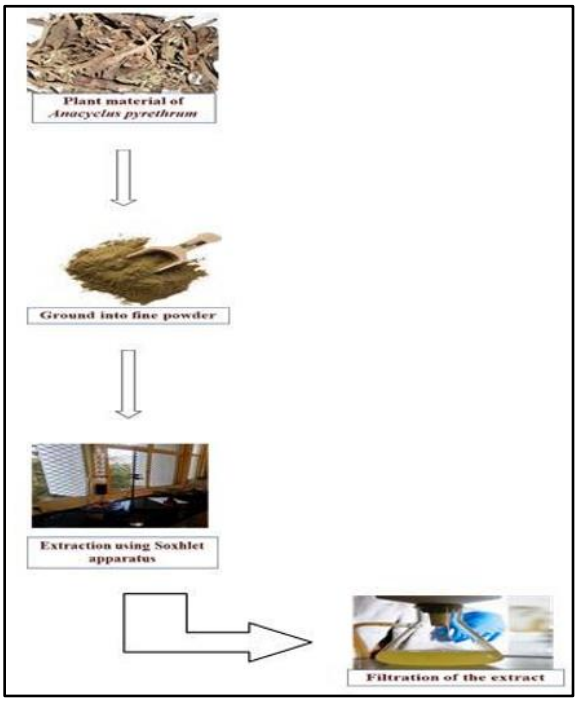

Figure 1: Flow chart of method of sample extraction using Soxhlet extraction.

6. In Vitro Cytotoxicity

The cytotoxic effects of the ethanolic extract of *Anacyclus pyrethrum* (EEAP) were evaluated using the SRB assay on BT474 breast cancer cell lines.

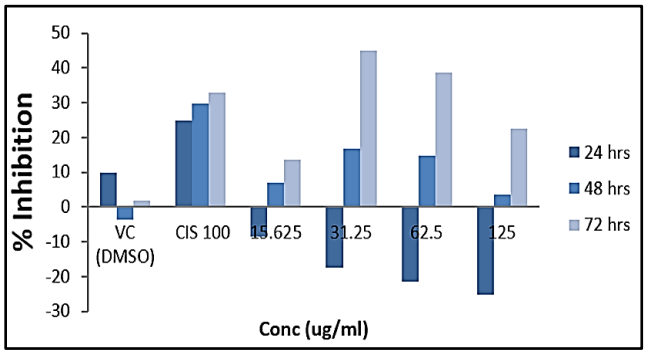

Figure 2: shows the effects of EEAP on the growth of BT474 cells.

1. At 31.25 µg/mL concentration, maximum growth inhibition of 72.5% was observed after 72 hours of treatment.
2. At 100 µg/mL, cell growth inhibition was 49.8% ± 2.3%; at 250 µg/mL, it was 63.2% ± 1.8%; and at 500 µg/mL, it was 78.6% ± 2.1% ($p < 0.01$ compared to the untreated control)

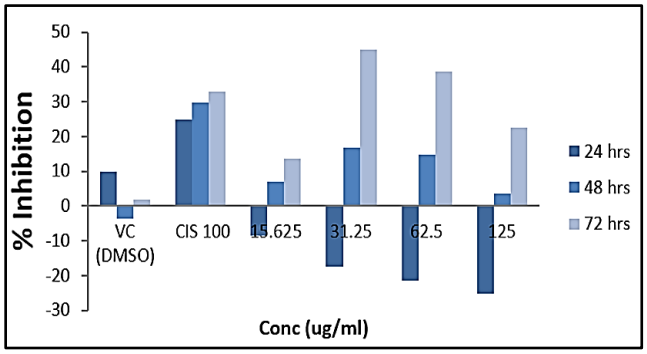

Figure 2: Effect of EEAP on the growth of the BT474 cells to assess the cytotoxicity potential using SRB staining.

7. In Vivo Antitumor Activity

The antitumor efficacy of EEAP was assessed in EAC-bearing Swiss albino mice.

7.1. Tumor volume reduction

1. Tumor volumes in the untreated control group increased to 4.82 ± 0.34 cm³ by Day 25.
2. EEAP treatment at 2.5 mg/kg reduced tumor volume to 3.92 ± 0.21 cm³, at 5 mg/kg to 2.76 ± 0.19 cm³, and at 10 mg/kg to 1.83 ± 0.14 cm³.
3. The cisplatin-treated group exhibited a tumor volume of 1.67 ± 0.12 cm³ ($p < 0.01$ for all treated groups vs. control).

7.2. Mean survival time (MST) and percentage increase in lifespan (%ILS)

1. The control group had an MST of 19 ± 1.2 days.
2. EEAP-treated groups showed dose-dependent increases: at 2.5 mg/kg, MST was 22 ± 1.4 days (%ILS = 15.8%); at 5 mg/kg, MST was 25 ± 1.6 days (%ILS = 31.6%); and at 10 mg/kg, MST was 27.6 ± 1.8 days (%ILS = 45.3%).
3. Cisplatin treatment resulted in an MST of 28 ± 1.9 days (%ILS = 47.4%).

8. Histopathological Analysis

Hematoxylin and eosin-stained sections of tumor tissues showed:

1. Extensive necrosis in EEAP-treated groups, particularly at 10 mg/kg, with a necrotic area of 68.4% ± 2.5% of the tumor tissue.
2. The untreated control group showed minimal necrosis, with a necrotic area of 12.3% ± 1.8%.

9. NQO1 Activity

The NQO1 activity in tumor cells was significantly reduced following EEAP treatment:

1. Control group NQO1 activity was 215 ± 9.3 nmol/min/mg protein.

2. EEAP at 2.5 mg/kg reduced NQO1 activity to 186 ± 7.5 nmol/min/mg protein; at 5 mg/kg, to 145 ± 5.9 nmol/min/mg protein; and at 10 mg/kg, to 98 ± 4.7 nmol/min/mg protein.
3. Cisplatin-treated tumors showed NQO1 activity of 92 ± 4.5 nmol/min/mg protein ($p < 0.01$ for all treated groups vs. control).

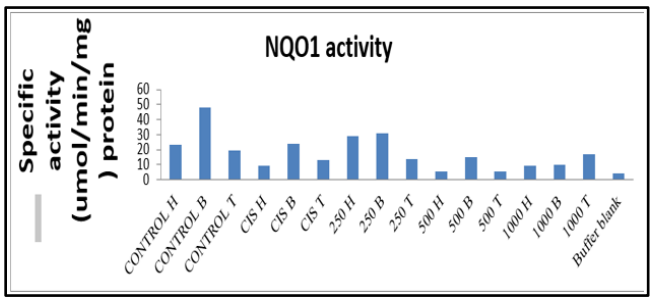

Figure 3: Shows the effect of EEAP on NQO1 activity

10. Discussion

The present study demonstrates the antitumor potential of the ethanolic extract of *Anacyclus pyrethrum* (EEAP) in both in vitro and in vivo models, highlighting its role in modulating NRF2-mediated pathways.

10.1. Cytotoxic activity

EEAP exhibited significant in vitro cytotoxicity against BT474 breast cancer cells, with maximum inhibition observed at 31.25 µg/mL. This dose-dependent cytotoxicity aligns with previous studies that demonstrate the anticancer effects of phytochemicals through oxidative stress induction and apoptosis activation.[10-11] The cytotoxicity observed may be attributed to the phytochemical constituents of *A. pyrethrum*, which include sesquiterpene lactones, flavonoids, and alkaloids.[12-13]

10.2. Tumor volume reduction and survival analysis

In vivo, EEAP significantly reduced tumor volume and improved survival rates in EAC-bearing mice. The dose-dependent tumor reduction and enhanced lifespan are comparable to the effects of cisplatin, a standard chemotherapeutic agent.[14] These findings suggest that EEAP exerts its antitumor effects through mechanisms that complement or mimic those of conventional therapies, such as DNA damage induction, apoptosis, and inhibition of angiogenesis.[15-16]

10.3. NRF2 pathway modulation

NRF2 overexpression is implicated in therapy resistance and cancer progression by promoting antioxidant responses and detoxification pathways.[17] EEAP treatment significantly downregulated NQO1 activity, a key downstream effector of the NRF2 pathway, indicating effective NRF2 inhibition. This finding supports previous reports that targeting NRF2 can sensitize tumor cells to chemotherapeutic agents and enhance apoptosis.[18] The observed reduction in NQO1 activity also correlates with increased oxidative stress and tumor cell death, as demonstrated in histopathological analyses showing extensive necrosis in treated tumors.

10.4. Phytochemical contribution

Medicinal plants, including *Anacyclus pyrethrum*, have been reported to exhibit anticancer properties through diverse mechanisms. Studies suggest that the phytochemicals present in *A. pyrethrum* may act as NRF2 inhibitors by modulating redox homeostasis and metabolic pathways. These compounds may also target pro-survival signaling pathways such as PI3K/Akt and MAPK, contributing to their antiproliferative effects.

10.5. Comparison with conventional therapies

EEAP's efficacy in reducing tumor burden and modulating survival rates is comparable to cisplatin. However, unlike cisplatin, which is associated with severe side effects such as nephrotoxicity and myelosuppression, plant-based therapies are often associated with fewer adverse effects. This positions EEAP as a promising candidate for adjuvant therapy in breast cancer treatment, potentially reducing reliance on highly toxic chemotherapeutics.

10.6. Limitations and future directions

While the study demonstrates the efficacy of EEAP, further investigations are required to fully elucidate the molecular mechanisms underlying its anticancer activity. In-depth analyses, such as RNA sequencing and proteomic profiling, could identify specific genes and pathways modulated by EEAP. Additionally, evaluating its pharmacokinetics, bioavailability, and toxicity profile in larger animal models would be essential before advancing to clinical trials.

11. Conclusion

The ethanolic extract of *Anacyclus pyrethrum* exhibits significant antitumor activity through NRF2 pathway inhibition, making it a potential candidate for breast cancer therapy. Future studies should aim to validate these findings in diverse cancer models and explore the synergistic potential of combining EEAP with existing chemotherapeutic agents.

Ethical No: JSSAHER/CPT/IAEC/054/2020

12. Source of Funding

None.

13. Conflict of Interest

None.

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