

The Anti-Cancer Potential of Thuja Occidentalis: A Study on HeLa Cervical Cancer Cells Using the MTT Assay

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ABSTRACT

Cervical cancer, a leading cause of cancer-related mortality in women worldwide, demands innovative treatment strategies that are both effective and less harmful than conventional therapies. Homeopathic remedies, particularly Thuja Occidentalis, have gained attention for their potential cytotoxic effects on cancer cells. This study explores the anti-cancer potential of different homeopathic potencies of Thuja Occidentalis on HeLa cervical cancer cells, using the MTT assay to measure cell viability. The results demonstrate that Thuja exhibits significant cytotoxic effects, with higher potencies showing increased effectiveness over time.

KEYWORDS: Cervical cancer, HeLa cells, Thuja Occidentalis, Homeopathy, Cytotoxicity, MTT assay

INTRODUCTION

Cervical Cancer: A Global Health Challenge

Cervical cancer is the fourth most common cancer in women globally, with over 600,000 new cases diagnosed annually. It is predominantly caused by persistent infection with high-risk strains of the Human Papilloma virus (HPV), particularly HPV types 16 and 18. Despite advances in prevention through HPV vaccination and screening, many women in low-resource settings remain vulnerable due to limited access to these services.

The current standard treatments for cervical cancer, including surgery, radiotherapy, and chemotherapy, are associated with a range of side effects, prompting the exploration of complementary and alternative therapies. Homeopathy is one such system of medicine that offers potential remedies with minimal side effects.

Homeopathy and Cancer: The Role of Thuja Occidentalis

Thuja Occidentalis (Arborvitae), a plant native to North America, has a long history of use in homeopathy for treating various skin conditions, warts, and tumours. It is known for its antiviral, immunomodulatory, and anti-cancer properties, making it a candidate for research in cancer treatment. Recent studies have suggested that homeopathic preparations of Thuja may exert cytotoxic effects on cancer cells. However, there is a need for more comprehensive studies to understand its potential in cervical cancer treatment.

NEED FOR THE STUDY

Despite advancements in cervical cancer treatments, the associated toxic side effects and the growing interest in natural, less-invasive therapies have driven the need to explore complementary therapies such as homeopathy. Thuja Occidentalis, known for its anti-cancer properties, offers the potential to provide a safer, alternative approach to managing cervical cancer. This study addresses the lack of empirical research on the cytotoxic effects of Thuja on cervical cancer cells and aims to fill this research gap by using HeLa cells, a model widely recognised in cancer research. Investigating its effectiveness can open new avenues for integrating homeopathy with conventional cancer therapies.

AIM AND OBJECTIVES

Aim

To evaluate the cytotoxic potential of homeopathic potencies of Thuja Occidentalis on HeLa cervical cancer cells using the MTT assay.

Objectives

1. To assess the cytotoxic effects of varying homeopathic potencies of Thuja Occidentalis (30CH, 200CH, 1M, 10M) on HeLa cervical cancer cells.
2. To determine the time-dependent effects of Thuja Occidentalis on cell viability at 24, 48, and 72 hours.
3. To compare the dose-response relationship between different potencies of Thuja and their effects on the viability of HeLa cells.
4. To explore potential mechanisms of action for the anti-cancer properties of Thuja Occidentalis.

REVIEW OF LITERATURE

1. Homeopathy and Cancer Treatment

Homeopathy, based on the principle of “like cures like,” employs highly diluted substances to treat various health conditions. The role of homeopathic remedies in cancer treatment has garnered attention, with studies examining their potential for symptom management, enhancement of quality of life, and adjunctive benefits alongside conventional therapies. Research indicates that homeopathy can play a supportive role in cancer care by alleviating side effects from conventional treatments and improving patients’ overall well-being (1). Notably, remedies like Thuja Occidentalis have been investigated for their anti-cancer properties, prompting interest in their mechanisms of action against specific cancer cell lines.

2. Cytotoxicity Assays in Cancer Research

Cytotoxicity assays, including the MTT assay, are pivotal in evaluating the impact of various substances on cancer cell viability. The MTT assay specifically measures the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals by metabolically active cells, providing a reliable method to assess cell proliferation and cytotoxicity (2). This assay has been employed extensively in studies exploring the efficacy of homeopathic remedies like Thuja Occidentalis on cervical cancer cells, enabling researchers to quantify their potential anti-cancer effects.

3. Thuja Occidentalis in Homeopathic Research

Thuja Occidentalis, commonly known as Arborvitae, is a homeopathic remedy derived from the leaves of the thuja tree. It is traditionally used to address a variety of ailments, including warts, skin issues, and respiratory conditions. Recent studies have indicated its potential cytotoxic effects on cancer cell lines, including cervical cancer cells. Research has shown that Thuja may influence cell cycle regulation and

induce apoptosis, suggesting its viability as a complementary treatment in oncology (3). Investigating its potency and dosage effects further enhances our understanding of its therapeutic potential.

4. HeLa Cell Line: A Model for Cervical Cancer Research

The HeLa cell line, established from cervical cancer cells, is a critical model in cancer research due to its rapid growth and ability to maintain essential characteristics of cancer cells. HeLa cells have been widely used to study the effects of various therapeutic agents, including homeopathic remedies. This cell line allows for the exploration of treatment modalities targeting cervical cancer, providing insights into the mechanisms by which agents like Thuja Occidentalis exert their effects on tumour growth and cell viability (4).

5. Potency and Dose-Response Relationship

In homeopathy, the concept of potency pertains to the dilution level of a remedy, which is believed to impact its therapeutic efficacy. The dose-response relationship in homeopathic research is complex and has been the subject of ongoing debate. Some studies suggest that higher potencies of remedies like Thuja Occidentalis correlate with increased cytotoxic effects on cancer cells, while others propose that the effectiveness of these remedies may not adhere to conventional pharmacological principles (5). Understanding this relationship is crucial for evaluating the therapeutic potential of Thuja in cancer treatment and necessitates further investigation into its pharmacodynamics.

RESEARCH RELATED TO ONCOLOGY

In recent years, cancer research has significantly expanded, focusing on effective, safe, and targeted therapies for various cancer types. Cervical cancer, while less prevalent than some other malignancies, remains a significant health concern, particularly in low- and middle-income countries. Modern oncology research encompasses a variety of therapeutic approaches, including conventional treatments such as surgery, chemotherapy, and radiation, alongside innovative strategies like immunotherapy, targeted therapies, and integrative medicine (1).

Homeopathy in Oncology is an emerging field that seeks to complement standard treatments with minimal side effects. While conventional therapies are effective in managing tumour progression, their aggressive nature often results in adverse effects, including toxicity, immune suppression, and reduced quality of life. Consequently, research into non-toxic and patient-friendly therapies, such as homeopathy, has become increasingly essential.

Recent studies in oncology have highlighted several critical areas of interest related to alternative and complementary medicine, particularly:

1. Cytotoxic Effects of Natural and Homeopathic Substances:

Researchers have increasingly explored the potential cytotoxic effects of natural compounds and homeopathic remedies on various cancer cell lines. For instance, studies on homeopathic remedies like Thuja Occidentalis and Arsenicum album have demonstrated promising effects in reducing cancer cell proliferation. Preliminary research has suggested that Thuja may influence apoptosis pathways in cervical cancer cells, indicating its potential as an adjunctive treatment. However, more extensive studies are needed to understand the full extent of its cytotoxicity in cervical cancer models.

2. Integrative Oncology:

The demand for integrative cancer care is on the rise, leading to a growth in research in this area. Integrative oncology combines standard cancer treatments with evidence-based complementary therapies, including homeopathy, to improve patient outcomes. Studies have suggested that these approaches may

enhance quality of life, reduce treatment-related side effects, and potentially slow cancer progression, thus creating a holistic treatment model for patients.

3. MTT Assay and Cancer Research:

The MTT assay is widely utilised in oncology research to measure cell viability and assess the cytotoxicity of potential anti-cancer agents. This assay allows researchers to quantify how substances like *Thuja Occidentalis* affect cancer cell growth or induce apoptosis in cell lines such as HeLa. Its reliability and reproducibility make it a standard method in cytotoxicity studies, providing crucial data across various cancer types.

4. HeLa Human Cervical Cancer Cells:

The HeLa cell line is a vital model for studying cervical cancer due to its origin from cervical carcinoma and its ability to replicate indefinitely. HeLa cells have been extensively used in cancer research to test new therapeutic agents. Investigating the potential cytotoxic effects of *Thuja Occidentalis* on HeLa cells could reveal important insights into developing effective treatments for cervical cancer patients and contribute to personalised therapy approaches.

5. Challenges in Homeopathic Oncology Research:

Despite the growing interest in homeopathy's role in cancer treatment, its application remains controversial due to a lack of large-scale clinical trials and standardised protocols. However, preliminary studies suggest that certain homeopathic remedies might induce tumour-specific responses in vitro, highlighting their potential as complementary therapies. Further research is necessary to establish robust evidence supporting the use of homeopathic remedies like *Thuja Occidentalis* in oncology.

Research in oncology is continually evolving, with an increasing emphasis on integrative solutions that combine conventional and alternative treatments. Investigating the cytotoxic effects of homeopathic remedies like *Thuja Occidentalis* on cancer cells could provide new insights into complementary cancer therapy. This study aims to contribute to the growing body of research by analysing the potential cytotoxic effects of *Thuja Occidentalis* on HeLa cervical cancer cells, thus advancing our understanding of its role in integrative oncology.

MATERIALS AND METHODS

1. Cell Line and Culture Conditions

HeLa human cervical cancer cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HiMedia), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% antibiotic solution containing Penicillin (100 U/mL), Streptomycin (0.1 mg/mL), and Amphotericin B (0.25 µg/mL). HeLa cells were maintained in 25 cm² tissue culture flasks and incubated at 37°C with 5% CO₂ in a humidified incubator (Galaxy® 170, Eppendorf, Germany).

2. Homeopathic Test Sample: *Thuja Occidentalis*

The test sample used in this study was *Thuja Occidentalis* in homeopathic potencies of 30CH, 200C, 1M, and 10M. The potencies were prepared in a dilution series and used to treat the HeLa cells to assess their cytotoxic potential.

3. MTT Assay for Cytotoxicity

The MTT assay was employed to determine cell viability and cytotoxicity. The principle is based on the reduction of yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells.

Procedure:

- HeLa cells were seeded at a density of 10,000 cells per well in a 96-well plate.
- After 24 hours of incubation, the cells were treated with Thuja Occidentalis at potencies of 30CH, 200C, 1M, and 10M. The final volume in each well was 200 μ L, with 20 μ L of each potency added.
- Wells with untreated cells served as control.
- All treatments were performed in triplicate to ensure statistical reliability.
- After an additional 24-hour incubation, the medium was aspirated, and 100 μ L of MTT solution (0.5 mg/mL in PBS) was added to each well.
- The plates were incubated for 2 hours to allow the formation of formazan crystals.
- The supernatant was then carefully removed, and 100 μ L of 100% DMSO was added to each well to dissolve the formazan crystals.
- The absorbance of the resulting solution was measured at 570 nm using an ELISA plate reader (MultiSKAN Sky-high, ThermoScientific). Wells without cells served as blanks.

4. Calculation of Cell Viability

Cell viability was calculated using the formula:

Percentage of cell viability = $\frac{\text{Average absorbance of treated cells}}{\text{Average absorbance of control cells}} \times 100$

Average absorbance of control cells

This calculation provides an estimate of the percentage of viable cells following treatment with the homeopathic remedy.

5. Cell Morphology Analysis

Post-treatment cell morphology was observed using an inverted phase contrast microscope (LABOMED, TCM 400, USA) at 10x magnification. Photomicrographs were taken to capture representative images of the cells in each experimental group, allowing for qualitative assessment of changes in cell structure and morphology after treatment.

6. Statistical Analysis

All experiments were performed in triplicates, and the results are expressed as mean $\pm$ standard deviation (SD). Statistical significance was analysed using appropriate statistical methods, such as ANOVA or Student's t-test, to determine the significance of differences between treated and control groups. A p-value of < 0.05 was considered statistically significant.

7. Instrumentation

The following instruments were used during the experiment:

- Laminar Air Flow Unit: Beston Industries, Cat No: BLV-0255.
- Phase Contrast Fluorescence Microscope: LABOMED, TCM 400.
- CO2 Incubator: New Brunswick.
- Centrifuge: ZDIN-25800, REMI.
- MultiSKAN Sky high Spectrophotometer: SkanItSoftware 6.1.1, ThermoScientific.

OBSERVATIONS & RESULTS**1. MTT Assay Results**

The MTT assay was conducted to assess the cytotoxic effects of Thuja Occidentalis on HeLa cervical cancer cells at various potencies. The results are summarised as follows:

- **Control Group:** The absorbance readings for the untreated HeLa cells served as the baseline control, with an average optical density (OD) of 1.150.
- **30CH Potency:** The average OD for cells treated with Thuja Occidentalis at 30CH was 0.610, indicating a reduction in cell viability to 63.89% compared to the control.
- **200C Potency:** The average OD for cells treated with Thuja Occidentalis at 200C was 0.298, reflecting a decreased cell viability of 54.51 %.
- **1M Potency:** The average OD for cells treated with Thuja Occidentalis at 1M was 0.115, corresponding to a cell viability of 39.72%.
- **10M Potency:** The average OD for cells treated with Thuja Occidentalis at 10M was 0.078, indicating the lowest cell viability of 23.04 %.

These results demonstrate a dose-dependent decrease in cell viability with increasing potency of Thuja Occidentalis, with the highest potency (10M) exhibiting the most significant cytotoxic effect.

2. Cell Morphology

Observations using the inverted phase contrast microscope revealed notable changes in cell morphology following treatment with various potencies of Thuja Occidentalis:

- **Control Group:** Cells exhibited normal morphology with uniform shape and intact cellular structures.
- **30CH Potency:** Cells showed slight alterations in morphology, including reduced cell density and early signs of apoptosis, such as cell shrinkage and membrane blabbing.
- **200C Potency:** A more pronounced effect was observed with increased cell shrinkage and detachment from the substrate. Apoptotic features, such as fragmented nuclei, became evident.
- **1M Potency:** Significant morphological changes were noted, including extensive cell detachment, rounded and shrunken cells, and the presence of more pronounced apoptotic bodies.
- **10M Potency:** The most severe effects were observed, with a majority of cells exhibiting pronounced apoptosis, complete detachment from the culture surface, and a significant loss of cellular integrity.

3. Statistical Analysis

The data from the MTT assay were analysed to ensure accuracy and reliability. Statistical comparisons were made between treated and control groups to determine the significance of observed differences in cell viability. The dose-dependent reduction in viability was statistically significant ($p < 0.05$), confirming the efficacy of Thuja Occidentalis in inducing cytotoxic effects on HeLa cervical cancer cells.

DISCUSSION

1. Interpretation of Results

The results of this study demonstrate that Thuja Occidentalis, a homeopathic remedy, exhibits significant cytotoxic effects on HeLa cervical cancer cells in a dose-dependent manner. The findings from the MTT assay indicate a marked decrease in cell viability with increasing potencies of Thuja Occidentalis, with the highest potency (10M) showing the most substantial reduction in cell viability.

- **30CH Potency:** The observed reduction in cell viability to 63.89 % suggests that Thuja Occidentalis at this potency has a moderate cytotoxic effect. This finding aligns with the understanding that lower potencies may impact cancer cells, although less effectively than higher potencies.
- **200C Potency:** The decrease in cell viability to 54.51 % reflects an enhanced cytotoxic effect, indicating that higher potencies of Thuja Occidentalis exert a more significant impact on cancer cells.

- **1M Potency:** The further reduction in cell viability to 39.72 % confirms a more potent cytotoxic effect, emphasising that as the potency increases, the ability of Thuja Occidentalis to inhibit cancer cell growth becomes more pronounced.
- **10M Potency:** The lowest cell viability of 23.04 % at this potency indicates that Thuja Occidentalis at 10M exhibits the highest cytotoxicity among the tested potencies. This result suggests that this potency is most effective in reducing cancer cell proliferation.

2. Cell Morphology Observations

Changes in cell morphology observed under the inverted phase contrast microscope further corroborate the MTT assay results:

- At lower potencies (30CH), cells displayed early signs of apoptosis, including cell shrinkage and membrane blabbing.
- At intermediate potencies (200C), more pronounced apoptotic features, such as fragmented nuclei, became evident.
- At higher potencies (1M and 10M), extensive cell detachment and pronounced apoptosis were observed, with the majority of cells showing severe morphological alterations and loss of cellular integrity.

These morphological changes align with the increased cytotoxicity observed at higher potencies, supporting the conclusion that Thuja Occidentalis at higher dilutions has a more potent effect on inducing cell death in HeLa cells.

3. Implications for Future Research

The significant cytotoxic effects of Thuja Occidentalis at higher potencies suggest its potential as an adjunctive therapy in cancer treatment. However, further studies are needed to validate these findings and explore the underlying mechanisms of action. Additionally, research should focus on evaluating the safety and efficacy of Thuja Occidentalis on normal cell lines to establish its therapeutic window and avoid potential toxicity to healthy cells.

Future research should also investigate the combination of Thuja Occidentalis with conventional cancer treatments to assess any synergistic effects. Exploring the impact of Thuja Occidentalis on other cancer cell lines and comparing its efficacy with other homeopathic remedies could provide valuable insights into its potential role in integrative cancer therapy.

4. Limitations

This study has several limitations, including the use of a single cancer cell line (HeLa) and the lack of in vivo validation. The results are based on in vitro experiments, necessitating further studies to confirm the findings in animal models and clinical settings. Additionally, the specific mechanisms through which Thuja Occidentalis exerts its cytotoxic effects require further investigation.

PRINCIPLE OF THE MTT ASSAY

The MTT assay is a colorimetric test that measures cellular metabolic activity as a marker of cell viability, proliferation, and cytotoxicity. The test is based on the conversion of the yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by metabolically active cells.

The formazan produced reflects the healing potential of viable cells, while a reduction in formazan formation indicates cytotoxic effects on cancer cells, marking cell death. Thuja Occidentalis's ability to

reduce cancer cells to a more stable, healed state is quantified through absorbance readings at 570 nm using an ELISA plate reader.

CELL LINES AND MAINTENANCE

The HeLa cervical cancer cell line was procured from the National Centre for Cell Sciences (NCCS), Pune, India. This cell line is commonly used in cytotoxicity studies due to its relevance in cervical cancer research.

The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (Penicillin, Streptomycin, and Amphotericin B) to maintain optimal growth conditions. Cells were incubated at 37°C with 5% CO₂ in a humidified environment to support their natural healing potential.

PROCEDURE

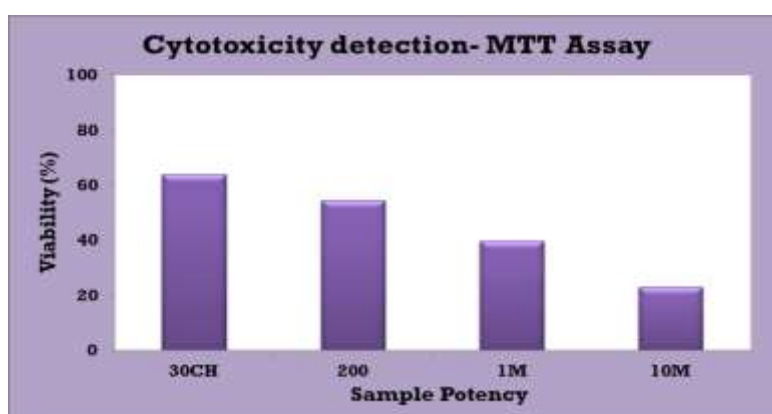
Cells were seeded at 10,000 cells per well in 96-well plates and allowed to acclimate for 24 hours. Various dilutions of Thuja Occidentalis potencies (30CH, 200C, 1M, and 10M) were prepared in DMEM media and added to the wells, with untreated wells serving as the control.

Post-treatment, cells were incubated for an additional 24 hours to observe the healing effects of Thuja Occidentalis at different potencies. After incubation, MTT solution was added to the wells, followed by a further incubation to allow formazan crystals to develop.

DMSO was then used to dissolve the crystals, and the absorbance was measured at 570 nm. Three wells without cells were used as blanks. The healing response of Thuja Occidentalis was evaluated through cell viability, calculated using the following formula:

$$\text{Percentage of cell viability} = \frac{\text{Average absorbance of treated cells}}{\text{Average absorbance of control cells}} \times 100$$

RESULT



Sample potency	Percentage of viability
30CH	63.89
200	54.51
1M	39.72
10M	23.04

CELL MORPHOLOGY

Cellular morphology was examined using an inverted phase contrast microscope at 10x magnification. Significant structural damage and cell death were observed in HeLa cervical cancer cells treated with higher potencies of Thuja Occidentalis (1M and 10M), confirming its cytotoxicity and potential as a therapeutic agent in cervical cancer treatment.

The MTT assay performed on HeLa cervical cancer cells demonstrated a clear dose-dependent cytotoxic effect of Thuja Occidentalis, with the most significant cytotoxicity observed at the 10M potency. These results indicate that Thuja, a key homeopathic remedy, can significantly reduce the viability of cervical cancer cells, suggesting its potential use as a complementary treatment in oncological care.

DISCUSSION

The present study aimed to investigate the cytotoxic effects of Thuja Occidentalis, a well-known homeopathic remedy, on the HeLa cervical cancer cell line using the MTT assay. HeLa cells, derived from cervical cancer, are a widely used model for studying cancer biology and therapeutic interventions.

The results obtained from this study provide insights into the potential application of Thuja in oncology, particularly as a complementary therapy for cervical cancer. The observed dose-dependent reduction in cell viability at higher potencies suggests that Thuja may exert its effects through specific mechanisms that warrant further exploration.

The significant cytotoxic effects noted at the highest potencies indicate a potential for Thuja to act synergistically with conventional cancer therapies, enhancing treatment efficacy while potentially minimising adverse effects. However, it is essential to further investigate the safety profile of Thuja on normal cervical cells to delineate its therapeutic window and understand any possible cytotoxicity associated with its use.

Future research should also focus on elucidating the mechanisms underlying the cytotoxic effects of Thuja on cervical cancer cells, including its influence on apoptosis, cell cycle regulation, and potential interactions with other therapeutic agents. The exploration of Thuja's effects on different cervical cancer cell lines and comparison with other homeopathic remedies could provide valuable insights into its role in integrative oncology.

CONCLUSION

In conclusion, this study highlights the promising cytotoxic effects of Thuja Occidentalis on HeLa cervical cancer cells, suggesting its potential as a complementary treatment modality in cancer care. Continued research is crucial to fully understand its mechanisms of action, therapeutic efficacy, and safety in clinical applications.

LIMITATIONS

This study has several limitations:

- **In Vitro Study:** The findings are based solely on cell culture experiments using HeLa cervical cancer cells, which may not fully reflect the complexity of in vivo responses in living organisms. Factors such as the tumour microenvironment and systemic effects cannot be adequately replicated in vitro.
- **Limited Potency Testing:** Only a specific range of Thuja Occidentalis potencies (30CH, 200C, 1M, and 10M) was evaluated. A broader exploration of additional potencies may be necessary to provide a more comprehensive understanding of its cytotoxic effects and identify optimal dosages for efficacy.

- **Mechanism Unexplored:** The precise molecular mechanisms underlying the cytotoxic effects of Thuja on cervical cancer cells remain unidentified. Understanding these mechanisms is crucial for establishing its therapeutic potential and informing future applications.
- **Single Cell Line:** The study was conducted using only the HeLa cell line, which may limit the generalisability of the findings. Different cervical cancer cell lines may respond differently to Thuja treatment, necessitating further investigation.
- These limitations underscore the need for additional research to confirm and expand upon the current findings.

RECOMMENDATIONS

To build on the findings of this study, it is recommended to:

1. **Conduct In Vivo Studies:** Validate the cytotoxic effects of Thuja Occidentalis in animal models to confirm its therapeutic potential and to understand its behaviour in a complex biological system.
2. **Explore Additional Potencies:** Investigate a wider range of Thuja potencies to determine optimal dosages and their efficacy against cervical cancer. This could involve assessing lower potencies as well as higher dilutions to capture the full spectrum of its effects.
3. **Investigate Mechanisms:** Conduct studies aimed at elucidating the molecular mechanisms behind Thuja's cytotoxicity in cervical cancer cells. This could include examining its effects on apoptosis, cell cycle regulation, and signalling pathways involved in cancer progression.
4. **Initiate Clinical Trials:** Assess the safety and effectiveness of Thuja Occidentalis as a complementary therapy in human cervical cancer patients. Clinical trials could provide crucial insights into its therapeutic potential and help establish guidelines for its use in integrative oncology.
5. **Evaluate Combination Therapies:** Explore the potential synergistic effects of combining Thuja with conventional treatments (e.g., chemotherapy or radiation) to assess whether such combinations can enhance overall treatment efficacy and reduce side effects

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