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Cytotoxicity Evaluation of *Thuja occidentalis* 6C on A549 Lung Cancer Cells: Insights into Hormesis and GC-MS Profiling

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Abstract: ***This study evaluates the cytotoxic effects of ethanolic Thuja occidentalis mother tincture and its serial dilutions (1C to 1M) on A549 human lung carcinoma cells. Cytotoxicity was measured using the MTT assay for the commercial mother tincture (MT) containing 90% ethanol and its subsequent dilutions. Among these, only the 6C dilution showed moderate inhibition of cell viability, while the mother tincture and other dilutions had minimal effects. This raises questions about whether the observed activity results from residual ethanol toxicity, which is known to interfere with viability assays, or from a hormetic response typical of ultra-dilute homeopathic preparations, where low doses stimulate biological effects, as described in toxicology. Results of ROS assays for the 6C dilution in 1.25% (v/v) ethanol support the involvement of oxidative stress–mediated mechanisms. The selective cytotoxicity observed only at the 6C dilution suggests a hormetic response rather than ethanol-induced toxicity. This aligns with emerging evidence that ultra-dilutions near 6C may retain nanoscale structures or physicochemical imprints capable of modulating oxidative stress pathways in cancer cells. Overall, the cytotoxic profile of Thuja occidentalis on A549 cells appears to be potency-dependent and warrants further investigation, particularly with a focus on nanoparticle characterization and redox-regulated signaling mechanisms.***

Keywords: *Thuja occidentalis*; Homeopathy, Hormesis; 6C dilution; A549 lung cancer cells

1. Introduction

Lung cancer remains one of the most prevalent and deadly forms of cancer globally, particularly affecting the lower respiratory tract. It is the leading cause of cancer-related deaths among men and women, with a five-year survival rate of merely 5% [1–4]. Among the various types of lung malignancies, adenocarcinomas account for approximately 31% [4]. The A549 cell line, derived from human alveolar basal epithelial cells, is widely used in lung cancer research. It was first developed by Giard et al. in 1972 [5]. While tobacco smoking is the main cause of lung cancer, environmental pollutants and airborne toxins also play a significant role in its development [6]. Despite the availability of many FDA-approved chemotherapeutic agents, such as Carboplatin-Taxol, Gemcitabine-Cisplatin, Methotrexate (Mexate), and Nivolumab, their use is often limited by severe side effects like leukopenia, neutropenia, myelosuppression, and respiratory issues [7,8]. This has directed current research toward finding safer, plant-based alternatives with fewer adverse effects.

Natural medicinal plants, which have long been used in traditional healing systems, are now being studied scientifically for their cytotoxic and therapeutic potential. *Thuja occidentalis* L., commonly called "white cedar," "tree of life" (arbor vitae), or simply "thuja," is an evergreen coniferous tree from the Cupressaceae family. It is native to Canada and North America and is widely cultivated in Europe, Asia, and India. In India, *Thuja occidentalis* is readily available and is often cultivated as both an ornamental and medicinal plant in gardens, herbal farms, and public parks, particularly in temperate and sub-tropical regions such as Himachal Pradesh, Uttarakhand, Jammu & Kashmir, and parts of the north-eastern states. The plant is also included in the Indian systems of medicine (AYUSH), especially in homeopathy and Unani medicine, due to its extensive pharmacological properties.

Traditionally, *T. occidentalis* (*Thuja occidentalis*) has been used to treat respiratory conditions such as bronchial catarrh, as well as urinary, reproductive, rheumatic, and autoimmune disorders, including cystitis, amenorrhea, and psoriasis [9]. In homeopathy, it is usually given as a mother tincture or in various diluted potencies [10]. The leaf branches, which are

rich in tannins, flavonoids, essential oils, and polysaccharides, are the most pharmacologically active parts of the plant. The plant's therapeutic potential is primarily ascribed to its essential oil, which contains diterpenes and monoterpenes such as α-thujone, β-thujone, fenchone, and sesquiterpenes.

Thujone ($C_{10}H_{16}O$), the main bioactive compound, makes up to 60% of the essential oil content and is found in a mixture of α- (more active) and β-isomers. These compounds contribute to Thuja's extensive pharmacological effects, including antioxidant, antitumor, antibacterial, antifungal, antiviral, antiulcer, antipsychotic, emmenagogic, diuretic, expectorant, and hepatoprotective activities [11]. Also, key constituents include α-pinene, α-thujone, 1-fenchone, borneol acetate, andisovaleric acid, terpineol, sabinene, camphor, valerianic camphoric acid, occidol, β-sitosterol, quercetin, rhodoxanthin, tannins, mucilage, and vitamin C. Among these, the main components of the essential oil from the leaves are α-pinene (34.4%), cedrol (13.17%), and phellandrene (8.04%). In contrast, the essential oil extracted from the cones mainly consists of α-pinene (58.55%) and 3-carene (24.08%). Notably, the leaf essential oil shows strong antioxidant activity, suggesting potential therapeutic applications for conditions related to oxidative stress. Thujone is a volatile monoterpene ketone, and GC-MS (Gas Chromatography-Mass Spectrometry) is the most appropriate analysis method.

2. Literature Survey

2.1 Hormesis as a Hypothetical Basis for Activity in Ultra-Dilute Homeopathic Preparations:

Hormesis is a dose–response phenomenon characterized by a biphasic response to a substance, where low doses stimulate or activate adaptive responses, while high doses inhibit or cause toxicity. In the context of ultra-diluted homeopathic preparations such as 6C dilutions, hormesis offers a potential mechanistic explanation for the paradoxical bioactivities observed, including selective cytotoxicity, redox imbalance, or metabolic interference. These effects may not follow traditional pharmacological dose-response curves but instead act via subtle biological modulations at the cellular or molecular level, especially in stress-sensitive systems like cancer cells.

The biological effectiveness of ultra-dilute homeopathic preparations, such as *Thuja occidentalis* 6C, remains a subject of debate in both complementary medicine and mainstream pharmacology. One plausible scientific explanation for these effects is the principle of hormesis, a biphasic dose–response pattern where low doses of a stressor or bioactive agent stimulate adaptive, often beneficial cellular responses. Conversely, higher doses lead to inhibitory or toxic effects [12].

In the context of homeopathy, hormesis provides a theoretical framework to explain the paradoxical biological activity seen in high-dilution preparations, even when there are no measurable amounts of the original substance. While traditional pharmacology often depends on dose-dependent effects, hormesis disputes this approach by proposing that ultra-low concentrations can act as mild stressors, activating compensatory mechanisms that improve cell survival, immune response, or metabolic regulation [13].

In cancer research, hormetic responses have also been documented in tumor and normal cells, where low-dose exposures to certain phytochemicals or oxidative agents modulate signaling pathways involved in apoptosis, ROS generation, and cellular repair. However, such responses can be dual-edged: while potentially protective in normal cells, they may enhance survival or resistance in cancer cells [14]. Therefore, when studying homeopathic dilutions in cancer models, it is crucial to consider whether observed cytotoxic or cytostimulatory effects arise from hormetic mechanisms, solvent artifacts (e.g., residual ethanol), or true bioactivity.

Thus, this study hypothesizes that the moderate cytotoxic effect observed with *T. occidentalis* 6C on A549 lung carcinoma cells may not be solely caused by residual ethanol or random variation, but could instead indicate a hormetic biological response triggered by ultra-dilute phytoconstituents. If confirmed, such findings could offer mechanistic insights into the controversial yet intriguing connection between homeopathy, hormesis, and anticancer research.

2.2 Hormesis: A Biphasic Dose–Response Phenomenon

In toxicology and pharmacology, hormesis challenges the conventional linear or threshold models of dose-response by suggesting that ultra-low doses of certain agents can activate adaptive stress responses, improve cellular functions, or modulate signaling pathways. Mechanistically, these low-dose stimulations may involve the activation of antioxidant defense systems, DNA repair enzymes, heat shock proteins, and other cytoprotective mechanisms [13]. In the context of homeopathy, hormesis provides a potential explanation for the biological activity of highly diluted preparations, such as centesimal dilutions (e.g., 6C). While controversial, proponents suggest that such ultra-dilutions may induce non-linear physiological responses, consistent with hormetic mechanisms. In cancer research, the concept of hormesis is particularly relevant when evaluating the cytotoxicity of natural products and dilute formulations, where low concentrations may paradoxically stimulate cellular viability or stress resistance, complicating the interpretation of results [14]. Therefore, distinguishing true bioactivity from solvent effects or experimental artifacts becomes critical, especially in assays like MTT or ROS measurements.

2.3 Key Mechanistic Areas

A hormetic biological response from *Thuja occidentalis* 6C on A549 cells shows the mechanistic insights to know how ultra-dilute preparations interact with cellular stress response pathways, particularly those related to cell survival, apoptosis**,** redox signaling**,** and gene expression. These key mechanistic areas are shown in Table 1.

Table 1: Mechanistic pathways potentially associated with hormesis induced by *T. occidentalis* 6C

Pathway/Mechanism	What to Investigate	Assays/Markers	Relevance to Hormesis
1. Oxidative Stress & Redox Balance	ROS production and antioxidant response	DCFDA assay, GSH levels, Nrf2, HO-1, SOD, Catalase	Mild oxidative stress may activate protective redox pathways
2. Apoptosis (Programmed Cell Death)	Induction or inhibition of apoptosis	Annexin V/PI, Caspase-3/-9, Bax/Bcl-2, p53	Hormetic doses may trigger adaptive apoptosis or survival
3. Cell Cycle Modulation	Arrest or stimulation of cell cycle progression	Flow cytometry, Cyclin D/E, p21, CDK4/6	Hormesis may cause cell cycle arrest or proliferation
4. Mitochondrial Function	Mitochondrial integrity and ATP production	JC-1 dye (ΔΨm), ATP assay, MitoSOX for mtROS	Central to apoptotic and stress responses
5. Stress Response Genes	Transcriptional activation of protective genes	qPCR, Western blot (HSP70, HSP90, NF-κB, IL-6)	Indicates cellular adaptation to ultra-low-dose stressors
6. Phytochemical-Specific Targets	Effect of thujone, borneol, etc. from GC-MS profiling	Targeted docking or pathway mapping based on identified compounds	Active constituents may mimic hormetic triggers

2.4 GC-MS study

GC-MS is a powerful analytical technique that combines the features of gas chromatography (GC) and mass spectrometry (MS) to identify and quantify substances within a sample. Commercial Thuja mother tincture. is typically a hydroalcoholic extract (often 60–90% ethanol). It contains polar compounds, non-volatile components, and other matrix elements not suitable for direct GC-MS injection [15, 16].

GC-MS is a highly sensitive and specific technique widely used for analyzing complex mixtures, including plant-based formulations such as homeopathic hydroalcoholic mother tinctures. In this context, GC-MS is a valuable tool for phytochemical profiling, allowing the separation, identification, and quantification of volatile and semi-volatile phytoconstituents. During analysis, the sample components are first separated in the gas chromatograph based on their interaction with the stationary phase and their volatility, each eluting at a characteristic retention time (RT). As compounds exit the GC column, they are ionized and fragmented in the mass spectrometer, generating mass spectra that are then matched against standard databases such as NIST or Wiley for compound identification. The retention time provides an initial identification, which is confirmed by the mass spectral pattern. The relative abundance of each phytoconstituent is calculated using the peak area normalization method, expressed as a percentage of the total ion chromatogram (TIC). This method facilitates the identification of key bioactive compounds and supports quality control, standardization, and batch-to-batch consistency in homeopathic formulations [17, 18]. Additionally, GC-MS analysis enhances understanding of therapeutic efficacy by revealing the presence and proportion of pharmacologically active constituents within the mother tincture.

3. Methodology

Materials for MTT assay, ROS, and Flow Cytometry:
The A549 human lung adenocarcinoma cell line (NCCS, Pune) was cultured in DMEM supplemented with 10% FBS (MP Biomedicals, Germany). Reagents, including DPBS, 0.25% Trypsin-EDTA, MTT, and cell culture–grade DMSO (Merck, Germany) were used. Cell culture was performed in T-25 flasks (Thermo Fisher Scientific, USA) and 96-well plates, with all procedures conducted under aseptic conditions in a Class II A2 Biological Safety Cabinet (Biobase, China). Incubation was done in a CO_2 incubator (Labwit, Australia), microscopy with an XDFL microscope (Sunny Instruments, China), and absorbance measurements using a microplate reader (Biobase, China).

MTT Assay Procedure:
Cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h to allow adherence. Twenty-two test samples were prepared out of which 11 commercial alcohol-based preparations [Thuja mother tincture (90% ethanol, v/v)] and their centesimal/millesimal dilutions] and their corresponding alcohol-free, water-adjusted equivalents. Cells were treated with test samples for 24 hours and 48 hours. Following treatment, MTT solution (0.5 mg/mL) was added and incubated for 3 h. Formazan crystals were dissolved in DMSO, and absorbance was recorded at 570 nm (reference 630 nm). Percentage cell viability and IC_{50} values were calculated from dose–response curves

ROS Assay Procedure:
A549 cells were seeded in 6-well plates (3×10^5 cells/well) and incubated overnight. After washing with PBS, cells were treated with test chemicals for 24 h, followed by incubation with H_2DCFDA (10 µM) for 1 h. Cells were trypsinized, centrifuged (300 × g, 5 min), washed twice with PBS, resuspended in 500 µl DPBS, and analyzed by flow cytometry (488 nm excitation, 525 nm emission).

Caspase-3 Assay (Flow Cytometry) Procedure:
Following treatment, cells were collected by combining culture medium, PBS wash, and trypsinized cells. After centrifugation (300 × g, 5 min) and PBS washes, cells were fixed in 2% paraformaldehyde (20 min), washed with 0.5% BSA in PBS, and processed for immunostaining.

Above 3 assays viz., MTT, ROS, and Caspase-3 were performed by Cell Kraft, Bengaluru, India.

GC-MS Procedure Using 90% Ethanol Mother Tincture (MT):
GC-MS, utilizing an Agilent 7890B gas chromatograph and a 5977B mass selective detector, driven by Chem-Station software, was used to investigate *Thuja occidentalis* alcoholic and water leaf extract. A DB-5 capillary column (30 m × 250 µm internal diameter × 0.5 µm film thickness) was used for component separation. Helium was used as the carrier gas, flowing at 1.0 mL/min with a 3 µL injection volume in split mode at a 10:1 split ratio. A 250°C inlet temperature was maintained. After 3 minutes at 40°C, the oven temperature

was increased at 7°C/min to 320°C for 10 minutes, completing the 50-minute protocol. In scan mode, the mass spectrometer covered 35–850 m/z. 230°C was the ion source temperature, while 150°C was the quadrupole temperature. Mass spectra were compared to NIST-20 Library entries to identify compounds. Only peaks with a match quality of 30% or higher were used for analysis. The methanolic extract yielded 17 bioactive compound peaks identified through CAS numbers, peak retention times, peak areas, heights, and mass spectral fragmentation patterns with known compounds from the NIST library.

Procedure for Testing 90% Ethanol Mother Tincture (MT) on Vero Cell Line and 6C granules on A549 cell line: The monolayer cell culture was trypsinized and adjusted to a density of 1×10^5 cells/ml in 10% FBS medium. A total of 100 µl of the diluted cell suspension (1×10^4 cells/well) was seeded into each well of a 96-well microtiter plate and incubated for 24 h to allow the formation of a partial monolayer. The supernatant was then removed, the monolayer was washed with medium, and 100 µl of various concentrations of the test compound/drug was added to the respective wells, followed by incubation at 37°C for 24 h in a 5% CO_2 atmosphere. After incubation, the test solutions were removed, and 100 µl of MTT solution (2 mg/ml in PBS) was added to each well. The plate was further incubated for 4 h at 37°C in a 5% CO_2 atmosphere. The supernatant was carefully discarded, and 100 µl of DMSO was added to solubilize the formazan crystals. The absorbance was measured at 570 nm using a microplate reader. The percentage of cell viability was calculated as:

%Viability=Absorbance of Sample/Absorbance of Control ×100

Dose–response curves were plotted for each cell line, and the IC_{50} value (concentration required to inhibit 50% of cell growth) was determined. GC–MS and Vero cell line assays using 90% ethanol-based mother tincture (MT) and assays on 6C granules of *Thuja* (without ethanol).

4. Results

The MTT assay was employed to assess and quantify cell viability in the A549 cell line using test samples containing alcohol and water. Table 1 illustrates MTT experiment outcomes, where purple coloration signifies live cells and a deep purple hue denotes increased cell viability. Light coloration signifies diminished viability and cytotoxicity. S1 to S6 (alcohol-based) over 24 hours across rows exhibits a lighter coloration, indicating less cell viability. In contrast, S7 to S12 (alcohol-based) and S13 to S22 (water-based) display moderate to deep purple coloration, suggesting enhanced viability at certain mid- to high dilutions. After 48 hours, in alcohol-based S1–S6, the purple intensity diminishes more than at 24 hours, indicating that prolonged exposure enhances cytotoxicity. Certain wells in S7–S11 (alcohol-based) and S12–S22 (water-based) exhibit lighter colors relative to the 24-hour set, while higher dilutions preserve greater colour. For alcohol-based samples (Table 2a), the 90% mother tincture showed the lowest cell vitality, followed by the 6C (57.38%) and 3C (61.31%) dilutions, with viability increasing at higher dilutions. For water-based samples (Table 2b), the lowest viability was observed for S12 (90% MT, 68.29%), followed by S21 (6M, 69.13%), while higher dilutions displayed comparatively greater cell viability.

Table 2 (a): Shows the MTT assays percentage cell viability results following a 24-hour exposure to alcohol-based samples from S1 to S11

			Control	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	Blank	Untreated	Control	90% MT	80% MT	1C	2C	3C	6C	30C	200C	1M	6M	10M
Reading 1	0.062	1.138	1.095	0.582	0.842	0.892	0.884	0.713	0.643	0.922	1.075	1.03	0.89	0.771
Reading 2	0.054	1.113	1.119	0.584	0.854	0.801	0.87	0.712	0.698	1.012	0.906	0.972	0.836	0.898
Mean OD	0.058	1.126	1.107	0.583	0.848	0.847	0.877	0.713	0.671	0.967	0.991	1.001	0.863	0.835
Mean OD-Mean Blank		1.0675	1.0490	0.5250	0.7900	0.7885	0.8190	0.6545	0.6125	0.9090	0.9325	0.9430	0.8050	0.7765
Standard deviation		0.0177	0.0170	0.0014	0.0085	0.0643	0.0099	0.0007	0.0389	0.0636	0.1195	0.0410	0.0382	0.0898
Standard error		0.0125	0.0120	0.0010	0.0060	0.0455	0.0070	0.0005	0.0275	0.0450	0.0845	0.0290	0.0270	0.0635
% Standard error		1.1710	1.1241	0.0937	0.5621	4.2623	0.6557	0.0468	2.5761	4.2155	7.9157	2.7166	2.5293	5.9485
% Viability		100	98.27	49.18	74.00	73.86	76.72	61.31	57.38	85.15	87.35	88.34	75.41	72.74

Table 2 (b) Shows the MTT assays percentage cell viability results following a 24-hour exposure to water-based samples from S12 (w) to S22 (w)

	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22
	90% MT (w)	80% MT (w)	1C (w)	2C (w)	3C (w)	6C (w)	30C (w)	200C (w)	1M (w)	6M (w)	10M (w)
Reading 1	0.806	0.908	1.182	0.934	1.025	1.024	1.065	1.007	1.093	0.856	1.054
Reading 2	0.768	1.034	1.134	0.891	0.997	0.985	0.974	0.944	1.071	0.736	0.934
Mean OD	0.787	0.971	1.158	0.913	1.011	1.005	1.020	0.976	1.082	0.796	0.994
Mean OD-Mean Blank	0.7290	0.9130	1.1000	0.8545	0.9530	0.9465	0.9615	0.9175	1.0240	0.7380	0.9360
Standard deviation	0.0269	0.0891	0.0339	0.0304	0.0198	0.0276	0.0643	0.0445	0.0156	0.0849	0.0849
Standard error	0.0190	0.0630	0.0240	0.0215	0.0140	0.0195	0.0455	0.0315	0.0110	0.0600	0.0600
% Standard error	1.7799	5.9016	2.2482	2.0141	1.3115	1.8267	4.2623	2.9508	1.0304	5.6206	5.6206
% Viability	68.29	85.53	103.04	80.05	89.27	88.67	90.07	85.95	95.93	69.13	87.68

After 48 hours, alcohol-based samples (Table 2c) showed the lowest cell viability with 90% MT (47.92%), followed by 3C (68.29%) and 6C (70.91%), while higher dilutions maintained moderate to high viability, reflecting an overall reduction compared to 24 hours. In water-based samples (Table 2d), viability was lowest for 30C (75.60%) and highest for 1C, with other dilutions showing comparatively higher viability.

Table 2 (c): Shows the MTT assays percentage cell viability results following a 48-hour exposure to alcohol-based samples from S1 to S11

			Control	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	Blank	Untreated	Control	90% MT	80% MT	1C	2C	3C	6C	30C	200C	1M	6M	10M
Reading 1	0.079	1.338	1.311	0.601	1.04	0.892	0.903	0.766	0.785	1.132	1.062	1.21	1.17	1.171
Reading 2	0.086	1.313	1.281	0.538	1.019	0.844	0.939	0.808	0.845	1.193	1.029	1.212	1.236	1.204
Mean OD	0.083	1.326	1.296	0.570	1.030	0.868	0.921	0.787	0.815	1.163	1.046	1.211	1.203	1.188
Mean OD-Mean Blank		1.2675	1.2380	0.5115	0.9715	0.8100	0.8630	0.7290	0.7570	1.1045	0.9875	1.1530	1.1450	1.1295
Standard deviation		0.0177	0.0212	0.0445	0.0148	0.0339	0.0255	0.0297	0.0424	0.0431	0.0233	0.0014	0.0467	0.0233
Standard error		0.0125	0.0150	0.0315	0.0105	0.0240	0.0180	0.0210	0.0300	0.0305	0.0165	0.0010	0.0330	0.0165
% Standard error		1.1710	1.4052	2.9508	0.9836	2.2482	1.6862	1.9672	2.8103	2.8571	1.5457	0.0937	3.0913	1.5457
% Viability		100	115.97	47.92	91.01	75.88	80.84	68.29	70.91	103.47	92.51	108.01	107.26	105.81

Table 2 (d): Shows the MTT assays percentage cell viability results following a 48-hour exposure to water-based samples from S12(w) to S22(w)

	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22
	90% MT (w)	80% MT (w)	1C (w)	2C (w)	3C (w)	6C (w)	30C (w)	200C (w)	1M (w)	6M (w)	10M (w)
Reading 1	1.039	1.185	1.184	1.033	1.013	1.096	0.84	1.121	1.011	0.912	0.962
Reading 2	1.105	1.126	1.142	0.978	1.089	1.127	0.89	1.166	0.959	0.842	1.107
Mean OD	1.072	1.156	1.163	1.006	1.051	1.112	0.865	1.144	0.985	0.877	1.035
Mean OD-Mean Blank	1.0140	1.0975	1.1050	0.9475	0.9930	1.0535	0.8070	1.0855	0.9270	0.8190	0.9765
Standard deviation	0.0467	0.0417	0.0297	0.0389	0.0537	0.0219	0.0354	0.0318	0.0368	0.0495	0.1025
Standard error	0.0330	0.0295	0.0210	0.0275	0.0380	0.0155	0.0250	0.0225	0.0260	0.0350	0.0725
% Standard error	3.0913	2.7635	1.9672	2.5761	3.5597	1.4520	2.3419	2.1077	2.4356	3.2787	6.7916
% Viability	94.99	102.81	103.51	88.76	93.02	98.69	75.60	101.69	86.84	76.72	91.48

4.1 Evaluation of Antioxidant and Anti-inflammatory Constituents in Thuja occidentalis Tincture Using GC-MS

The GC-MS analysis of *Thuja occidentalis* mother tincture revealed a diverse array of volatile and semi-volatile phytochemicals, many of which are known for their antioxidant and anti-inflammatory properties. While essential oils such as α-thujone and β-thujone were prominent, several other compounds contributed to the tincture's therapeutic potential. (Table 1)

4.1.1 Antioxidant Constituents

GC-MS analysis of *Thuja occidentalis* tincture revealed several antioxidant compounds. Guaiacol and eugenol are potent radical scavengers, while vanillin derivatives offer moderate protection against oxidative stress. β-Caryophyllene and humulene, both sesquiterpenes, activate antioxidant pathways and reduce ROS. Trace flavonoids like quercetin and kaempferol, possibly present in ethanolic extracts, further contribute to antioxidant effects.

4.1.2 Anti-inflammatory Constituents

The key anti-inflammatory constituents identified include α- and β-thujone, which act as COX-2 inhibitors; camphor, which reduces nitric oxide and prostaglandin levels; and fenchone/borneol, which modulate inflammatory enzymes. Linalool and terpineol exert their effects by suppressing pro-inflammatory cytokines through NF-κB inhibition, while methyl salicylate, if present, may provide aspirin-like activity. Collectively, these bioactives contribute to the tincture's therapeutic potential in mitigating oxidative stress and inflammation, consistent with its traditional medicinal applications.

4.1.3 Antioxidant Constituents

The antioxidant profile comprises guaiacol (2-methoxyphenol), a phenolic compound with potent radical scavenging capabilities that neutralizes reactive oxygen species (ROS) and alleviates oxidative stress; eugenol, a recognized antioxidant in plant essential oils that stabilizes free radicals and inhibits lipid peroxidation in cell membranes; and vanillin along with vanillic acid derivatives, which exhibit moderate antioxidant properties by modulating oxidative enzymes and averting cellular damage. Furthermore, β-caryophyllene, a bicyclic sesquiterpene, demonstrates significant antioxidant properties via Nrf2 pathway activation and interaction with CB2 receptors, whereas humulene, another sesquiterpene, mitigates reactive oxygen species and regulates oxidative indicators in inflammatory pathways. Trace amounts of flavonoid aglycones, like quercetin and kaempferol, which may be identified in ethanol-based extractions, additionally enhance the antioxidant properties. The anti-inflammatory components comprise α-thujone and β-thujone, monoterpenes exhibiting COX-2 inhibitory properties and moderate suppression of pro-inflammatory cytokines at therapeutic levels; camphor, which inhibits nitric oxide production and prostaglandin synthesis; and fenchone and borneol, which regulate inflammatory enzymes and decrease vascular permeability. Linalool, a monoterpenoid alcohol, diminishes TNF-α and IL-6 expression through the suppression of the NF-κB pathway, whereas terpineol attenuates nitric oxide and cytokine production in activated macrophages. Methyl salicylate, when present, demonstrates significant anti-inflammatory properties via inhibiting prostaglandin formation, akin to aspirin. The presence of these compounds in *Thuja occidentalis* tincture supports its reported antioxidant and anti-inflammatory effects. Their identification through GC-MS analysis not only validates traditional therapeutic claims but also highlights the formulation's potential in oxidative stress-related and inflammatory conditions.

4.1.4 Cytotoxicity Measurement

The cytotoxic potential of *Thuja occidentalis* mother tinctures, prepared using ethanol and water as solvents, was assessed on A549 human lung carcinoma cell lines using the standard MTT assay protocol. This colorimetric assay measures cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. The assay is based on the reduction of the yellow tetrazolium salt, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide], into insoluble purple formazan crystals by the mitochondrial dehydrogenase enzymes of metabolically active cells.

Figure 1, demonstrates that treatment with 6C granules reduced the viability of A549 lung cancer cells in a dose-dependent manner, resulting in an IC_{50} value of 32.67%. Figure 2, illustrates the effect of the 90% mother tincture on Vero cells, with a concentration of 100% yielding an IC_{50} value of 34.12%.

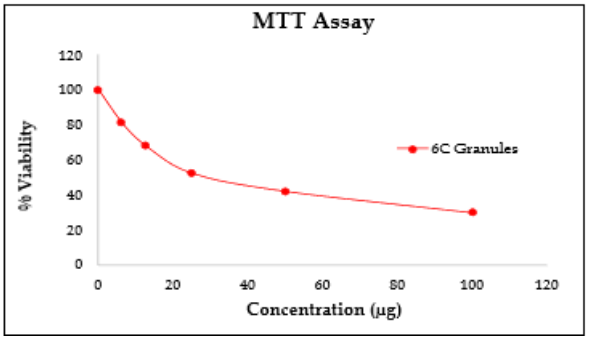

Figure 1: Graph of percentage cell viability versus concentration of 6C granules on A549 cell line, showing an IC_{50} value of 32.67%

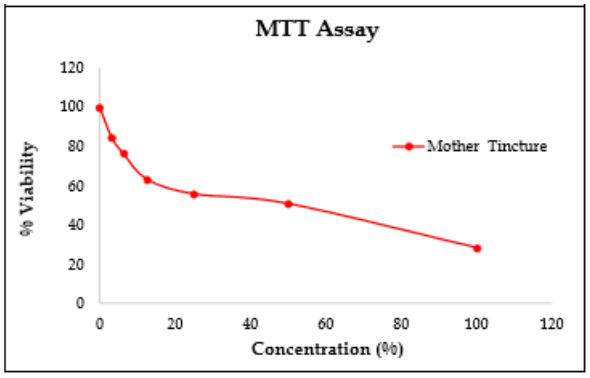

Figure 2: Graph of percentage cell viability versus concentration of 90% mother tincture on Vero cell line, showing an IC_{50} value of 34.12% within the tested concentration range (up to 100%)

4.1.5. ROS Assay

Table 3, indicates that untreated control cells exhibited a significant proportion of ROS-low cells (90.9% and 88.7% across two trials) alongside comparatively low ROS-high populations (9.1% and 11.3%). Conversely, treatment with Thuja 6C (1.25% v/v) significantly elevated the ROS-high population to approximately 25.7–25.8%, accompanied by a similar decrease in ROS-low cells to about 74%. The geometric mean fluorescence intensity (MFI) corroborated this, rising from 528–676 in control samples to 1146–1148 in treated samples, thereby demonstrating elevated intracellular ROS levels.

Table 3: Reactive oxygen species (ROS) assay of 6C sample, showing percentage of ROS-high and ROS-low cells with corresponding mean fluorescence intensity (MFI)

S. No	Sample Name		% of Cells		Geometric Mean Fluorescence intensity (MFI) of DFCDA (FL-1-A Parameter)
			ROS Low	ROS High	
1	Control (untreated)	Run 1	90.9	9.1	528
		Run 2	88.7	11.3	676
2	S6- 6C- 1.25% v/v	Run 1	74.3	25.7	1148
		Run 2	74.2	25.8	1146

The bar graph illustrates a significant increase in mean fluorescence intensity in *Thuja* 6C-treated cells (~1200) compared to the control (~600). This indicates enhanced intracellular ROS generation following treatment as shown in Figure 3.

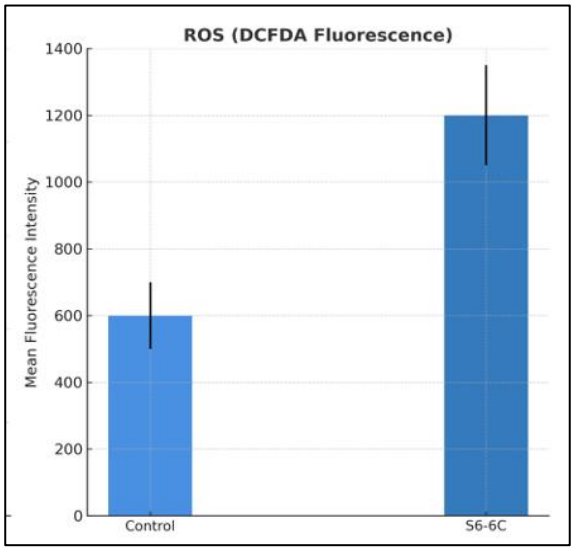

Figure 3: Reactive oxygen species (ROS) assay of 6C sample showing percentage of ROS-high and ROS-low cells with corresponding mean fluorescence intensity (MFI)

4.1.6 Caspace-3 Assay

The untreated control group displayed 3.4–4.9% of Caspase-3 positive cells, with mean fluorescence intensity (MFI) values ranging from 770 to 825. Conversely, Thuja 6C therapy markedly diminished Caspase-3 positive to merely 0.4–0.7%, accompanied by reduced MFI values of 490–523. This signifies the inhibition of caspase-3 activation in treated cells relative to the control group as shown in Table 4.

Table 4: Effect of Thuja 6C (1.25% v/v) on caspase-3 activation in A549 cells

S. No	Sample Name		% of Cells		Geometric Mean Fluorescence intensity (IMFI) of FITC Caspase- 3 (FL-1-A Parameter)
			Caspase- 3 -ve	Caspase- 3 +ve	
1	Control (untreated)	Run 1	95.1	4.9	825
		Run 2	96.6	3.4	770
2	S6- 6C- 1.25% v/v	Run 1	99.6	0.4	490
		Run 2	99.3	0.7	523

The bar graph (Fig. 4) illustrates a significant reduction in mean fluorescence intensity of active Caspase-3 in cells treated with Thuja 6C (~500) relative to the untreated control (~800), showing diminished caspase-3 activation.

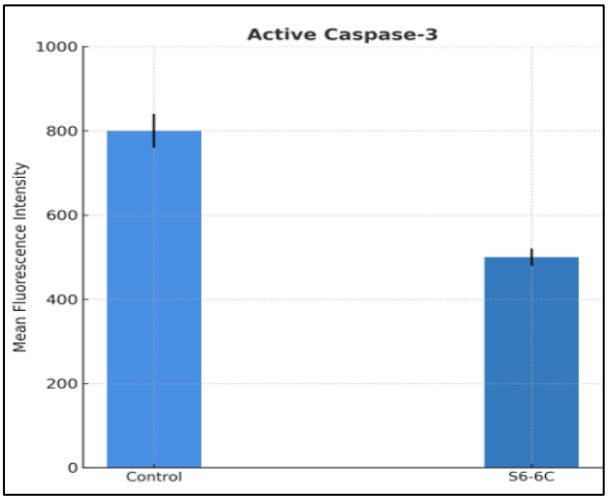

Figure 4: Effect of *Thuja* 6C (1.25% v/v) on Caspase-3 activation in A549 cells

5. Discussion

Our findings of moderate cytotoxicity of *Thuja occidentalis* 6C against A549 lung cancer cells parallel earlier work by Pathak et al. [19], where *Ruta graveolens* 6C selectively affected glioma cells while sparing normal lymphocytes. While these plant sources differ, both studies suggest that 6C potencies may elicit measurable biological effects in cancer cell models, underscoring the need to investigate their physicochemical characteristics and underlying mechanisms. From a hormesis perspective, such effects may reflect a biphasic dose response, where ultra-dilute preparations cause cell-type-specific stimulatory or inhibitory outcomes. In both Thuja and Ruta contexts, the 6C potency might fall within an "active window" that triggers adaptive stress responses, possibly involving oxidative stress regulation, mitochondrial disturbance, cell cycle arrest, or apoptosis while promoting protective pathways in normal cells. These responses are not easily explained by bulk phytochemicals alone, and emerging hypotheses suggest nano-scale structures or particle remnants as potential mediators. Viewing these results through a hormetic model provides a plausible, testable foundation for understanding selective cytotoxicity in ultra-dilute systems.

In our GC–MS analysis of Thuja occidentalis mother tincture (90% ethanol) and the 1.25% ethanolic preparation, we did not detect α-thujone or β-thujone, compounds often reported as key bioactives with anticancer potential. Several factors could explain this absence. First, thujone is highly volatile and thermolabile. During sample preparation and GC–MS injection, it can evaporate or degrade thermally, especially under the high injector and column temperatures used in GC analysis. Second, the chemical composition of Thuja preparations varies based on plant source, harvest time, and extraction conditions, such as differences in cultivar, growth environment, or processing, which can lead to low or undetectable thujone levels. Third, the efficiency of ethanol–water extraction for thujone depends on the balance of polarity. However, 90% ethanol generally extracts essential oils well; Post-preparation handling and storage can lead to oxidation or transformation into other terpenoid derivatives. Finally, in homeopathic preparations, serial dilution and succussion may further lower phytochemical concentrations below GC–MS detection limits, even if nano-scale remnants persist. Therefore, the absence of detectable thujone does not exclude biological activity, as other terpenoids, flavonoids, or nano-particulate plant remnants could contribute to the observed cytotoxic effects.

The present study demonstrates that *Thuja occidentalis* 6C exerts moderate cytotoxic effects on A549 human lung carcinoma cells. This finding is consistent with earlier observations by Pathak et al, where *Ruta graveolens* 6C displayed selective cytotoxicity against glioma cells. Despite originating from different botanical sources, both studies report significant activity specifically at the 6C potency, suggesting a potential shared mechanism that merits further investigation into the physicochemical and biological behaviour of ultra-diluted preparations.

Treatment with *T. occidentalis* 6C resulted in a marked increase in intracellular reactive oxygen species (ROS) levels compared to untreated controls. The percentage of ROS-high cells rose from approximately 10% in control samples to ~25% in treated groups, while mean fluorescence intensity (MFI) values more than doubled (from ~528–676 to ~1146–1148). These results were highly consistent across two independent experimental runs, underscoring the reproducibility of the observed effect. Biologically, such elevated ROS levels are known to induce oxidative stress, impair mitochondrial function, and initiate apoptotic signaling, suggesting a pro-oxidant cytotoxic mechanism for the 6C potency.

Interestingly, caspase-3 activity, commonly associated with classical apoptosis, was reduced in 6C-treated samples. The proportion of caspase-high cells dropped from 4.9% and 3.4% in controls to just 0.4% and 0.7% following treatment, accompanied by a reduction in MFI from ~825–770 (control) to ~490–523 (treated). This attenuation of caspase activation, despite elevated ROS levels, implies that *T. occidentalis* 6C may induce a caspase-independent form of cell death or trigger early mitochondrial dysfunction without fully engaging the canonical apoptosis cascade. While 6C is a high-dilution preparation, residual ethanol, standardized across all test samples to 1.25% (v/v), could theoretically contribute to cytotoxicity. However, the fact that mother tincture, 1C, 3C, 200C, and 1M dilutions did not produce similar effects suggests that ethanol alone is unlikely to explain the unique cytotoxic profile of 6C. Literature indicates that mitochondrial dysfunction and apoptosis in A549 cells can occur at ethanol concentrations ≥1%, especially when combined with bioactive extracts (e.g., from *Tremella mesenterica* or *Artemisia vulgaris*), but our experimental ethanol level remains within the non-toxic range [20].

5.1 Mechanistic Considerations and Hormesis Hypothesis

The selective cytotoxicity observed exclusively at the 6C dilution, despite uniform ethanol content across all samples, points toward a hormetic, non-linear dose–response phenomenon. In this model, an intermediate dilution like 6C elicits a biological effect not observed at higher or lower concentrations. This paradoxical activity aligns with hormesis theory and reflects findings in other ultra-dilute systems where low doses trigger adaptive or stress-related cellular responses. Emerging research suggests that certain homeopathic dilutions, particularly around the 6C potency, may retain source-derived nanoparticles or silica-associated molecular imprints formed during serial dilution and succussion. These nano-architectures, though present in ultra-trace amounts, may still interact with redox-sensitive cellular pathways, contributing to the observed elevation in ROS. Moreover, it is plausible that the 6C preparation achieves a unique physicochemical state, such as an optimal nanoparticle size, surface charge, or energy configuration, which facilitates cellular uptake or membrane interaction.

The absence of cytotoxicity at both lower (1C, 3C) and higher (200C, 1M) potencies further supports this hypothesis. To elucidate these phenomena, future studies should include nanoparticle characterization using techniques like transmission electron microscopy (TEM), scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX), dynamic light scattering (DLS), and zeta potential analysis. Metabolomic profiling and mitochondrial

function assays could also clarify the downstream effects of ROS elevation and determine whether the response reflects apoptosis, autophagy, necrosis, or other stress-mediated pathways.

6. Conclusion

This study demonstrates that *Thuja occidentalis* 6C exhibits selective cytotoxicity toward A549 lung cancer cells, characterized by a marked elevation in reactive oxygen species (ROS) and a reduction in cell viability, despite all tested liquid dilutions containing an identical ethanol concentration (1.25% v/v). The absence of comparable effects at other potencies, including the mother tincture, and the reproducibility of cytotoxic activity with ethanol-free 6C granules ($IC_{50} \approx 32.67$ µg/mL) indicate that the effect is not attributable to ethanol and may be an intrinsic property of the 6C preparation itself. Additionally, cytotoxicity testing of the 90% mother tincture on non-malignant Vero cells showed only moderate effects ($IC_{50} \approx 34–45\%$ v/v), further supporting that ethanol is not the primary driver of the observed cytotoxicity. This reinforces the possibility of a non-linear, hormetic dose–response uniquely associated with this potency. The observed decrease in caspase-3 activity, concurrent with elevated ROS, suggests a caspase-independent cell death pathway, potentially mediated through oxidative stress–driven mechanisms. These findings align with emerging hypotheses that ultra-dilute preparations may retain nano-scale physicochemical features capable of modulating cellular pathways. To substantiate these results and clarify their therapeutic significance, future research will focus on nanoparticle characterization and detailed analyses of mitochondrial dysfunction and redox-regulated signaling pathways.

7. Future Scope

Future research should concentrate on characterizing the nano-scale characteristics of Thuja occidentalis 6C through sophisticated methodologies, while concurrently examining mitochondrial dysfunction, redox-regulated signaling, and caspase-independent pathways. Extensive testing across many cancer and normal cell lines, coupled with in vivo validation, will facilitate the determination of selectivity, effectiveness, and safety. Furthermore, investigating synergistic interactions with conventional chemotherapeutics may elucidate its translational potential.

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References

[1] Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians*, 69(1), 7–34. https://doi.org/10.3322/caac.21551
[2] Hou, Z.-B., Lu, K.-J., Wu, X.-L., et al. (2014). In vitro and in vivo antitumor evaluation of berbamine for lung cancer treatment. *Asian Pacific Journal of Cancer Prevention*, 15(4), 1767–1769.
[3] Sihoe, A. D. L., & Yim, A. P. C. (2004). Lung cancer staging. *Journal of Surgical Research*, 117(1), 92–106.
[4] Beadsmoore, C. J., & Screaton, N. J. (2003). Classification, staging and prognosis of lung cancer. *European Journal of Radiology*, 45(1), 8–17.
[5] Giard, D. J., Aaronson, S. A., Todaro, G. J., et al. (1973). In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *Journal of the National Cancer Institute*, 51(5), 1417–1423.
[6] Brenner, D. R., et al. (2019). Alcohol and lung cancer risk among never smokers: A pooled analysis from the International Lung Cancer Consortium and the synergy study. *Cancer Epidemiology*, 58(2), 25–32.
[7] Li, A., et al. (2017). Docetaxel versus docetaxel plus cisplatin for non-small-cell lung cancer: A meta-analysis of randomized clinical trials. *Oncotarget*, 8(34), 57365–57378.
[8] Graziani, A., et al. (2017). Helmet continuous positive airways pressure (CPAP) treatment in a non-small cell lung cancer (NSCLC) patient with severe hypoxemic respiratory failure due to gemcitabine therapy. *European Journal of Oncology*, 22(1), 43–46.
[9] Alves, L. D. S., Figueirêdo, C. B. M., Silva, C. C. A. R., Marques, G. S., Ferreira, P. A., Soares, M. F. R., Silva, R. M. F., & Rolim-Neto, P. J. (2014). *Thuja occidentalis* L. (Cupressaceae): Review of botanical, phyto-chemical, pharmacological and toxicological aspects. *International Journal of Pharmaceutical Sciences and Research*, 5, 1163–1177.
[10] Naser, B., Bodinet, C., Tegtmeier, M., & Lindequist, U. (2005). *Thuja occidentalis* (Arbor vitae): A review of its pharmaceutical, pharmacological and clinical properties. *Evidence-Based Complementary and Alternative Medicine*, 2(1), 69–78.
[11] Chang, L. C., Song, L. L., Park, E. J., Luyengi, L., Lee, K. J., Farnsworth, N. R., Pezzuto, J. M., & Kinghorn, A. D. (2000). Bioactive constituents of *Thuja occidentalis*. *Journal of Natural Products*, 63(9), 1235–1238.
[12] Calabrese, E. J., & Baldwin, L. A. (2002). Defining hormesis. *Human & Experimental Toxicology*, 21(2), 91–97. https://doi.org/10.1191/0960327102ht217oa
[13] Mattson, M. P. (2008). Hormesis and disease resistance: Activation of cellular stress response pathways. *Human & Experimental Toxicology*, 27(2), 155–162. https://doi.org/10.1177/0960327107086170
[14] Tang, S., Zhang, Q., Wang, C., et al. (2021). The hormetic dose-response mechanisms: Nrf2 activation. *Pharmacological Research*, 167, 105526. https://doi.org/10.1016/j.phrs.2021.105547
[15] Rao, M. U., Ramesh, M., & Kumari, V. S. (2016). GC–MS analysis of bioactive compounds from the methanolic extract of *Wedelia trilobata* (L.) leaves. *Journal of Pharmacognosy and Phytochemistry*, 5(2), 324–328.
[16] Shrivastava, N., Patel, T., & Verma, S. (2014). Application of GC-MS for identification of bioactive constituents from plant extracts. *Current Trends in Biotechnology and Pharmacy*, 8(3), 326–336.

[17] Saxena, M., Saxena, J., Nema, R., Singh, D., & Gupta, A. (2013). Phytochemistry of medicinal plants. *Journal of Pharmacognosy and Phytochemistry*, 1(6), 168–182.
[18] Fiehn, O. (2002). Metabolomics – the link between genotypes and phenotypes. *Plant Molecular Biology*, 48(1–2), 155–171. https://doi.org/10.1023/A:1013713905833
[19] Pathak, S., Multani, A. S., Banerji, P., & Banerji, P. (2003). Ruta 6 selectively induces cell death in brain cancer cells but proliferation in normal peripheral blood lymphocytes: A novel treatment for human brain cancer. *International Journal of Oncology*, 23(4), 975–982. PMID: 12963976.
[20] Alli, A. A., Brewer, E. M., Montgomery, D. S., Ghant, M. S., Eaton, D. C., Brown, L. A., & Helms, M. N. (2014). Chronic ethanol exposure alters the lung proteome and leads to mitochondrial dysfunction in alveolar type 2 cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology*. https://doi.org/L-00287-2013