

In Vitro Evaluation of the Cytotoxic and Anti-Proliferative Potential of *Copaiva officinalis* (Mother Tincture and 6C Potency) Against PC3 Prostate Cancer Cells

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Abstract

Background And Objective: Prostate adenocarcinoma represents one of the leading malignancies affecting males globally. Given the limitations and toxicities associated with conventional androgen deprivation and chemotherapeutic regimens, natural and ultra-diluted pharmacological agents are under active investigation. To investigate and compare the in vitro anti-cancer, anti-proliferative, and cytotoxic effects of *Copaiva officinalis* in Mother Tincture (MT) and 6C dynamized potencies against the human androgen-independent PC3 prostate carcinoma cell line.

Materials and Methods: PC3 cells were treated with serial concentrations (0.5 to 64 $\mu\text{L/mL}$) of *Copaiva officinalis* MT and 6C alongside corresponding vehicle/dispersing alcohol controls (Control DA) across incubation periods of 24, 48, and 72 hours. Cytotoxicity was quantified via the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay to establish IC_{50} thresholds. Cell membrane integrity and viability kinetics were determined using the Trypan Blue exclusion assay.

Results: *Copaiva officinalis* MT elicited significant, concentration- and time-dependent cytotoxicity against PC3 cells, achieving maximal cytotoxicity of 79.47% at 24 hours, 93.86% at 48 hours, and 97.58% at 72 hours (64 $\mu\text{L/mL}$). The half-maximal inhibitory concentration (IC_{50}) for MT was 1.41 $\mu\text{L/mL}$ (24 h), 0.595 $\mu\text{L/mL}$ (48 h), and 0.45 $\mu\text{L/mL}$ (72 h), markedly outperforming vehicle controls. Conversely, *Copaiva officinalis* 6C demonstrated lower antiproliferative activity (IC_{50} of 28.85 $\mu\text{L/mL}$ at 24 h, 41.55 $\mu\text{L/mL}$ at 48 h, and 42.95 $\mu\text{L/mL}$ at 72 h), tracking closely with vehicle alcohol controls. Trypan Blue

exclusion assays corroborated these findings, where MT maintained persistent growth inhibition over 72 hours.

Conclusion: *Copaiva officinalis* Mother Tincture demonstrates pronounced cytotoxic and anti-proliferative activity against PC3 prostate cancer cells in vitro, whereas the dynamized 6C preparation exhibits attenuated direct cellular toxicity. Further exploration into the phytoconstituent mechanisms and apoptotic pathways of the mother tincture is warranted.

Keywords: *Copaiva officinalis*, Prostate cancer, PC3 cell line, MTT assay, Mother Tincture, Homeopathic dilutions, Cytotoxicity, In vitro oncology

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Introduction

Prostate carcinoma represents a major global health burden, ranking as the second most prevalent solid organ malignancy diagnosed in men and the sixth leading cause of male cancer deaths worldwide. According to epidemiological data from GLOBOCAN, over 1.4 million incident cases and approximately 375,000 deaths were recorded globally in 2020 alone.¹ Risk factor profiling identifies advancing age (particularly over 65 years), ethnicity (with higher incidence and disease severity in African American populations), familial genetic predisposition (including *BRCA1/BRCA2* mutations and Lynch syndrome), and westernized dietary patterns high in saturated fats as significant factors influencing disease emergence and progression.²

Standard management protocols for localized prostate tumors incorporate radical prostatectomy, external beam radiotherapy, and active surveillance. In advanced, recurrent, or metastatic stages, androgen deprivation therapy (ADT) remains the foundational intervention.³ However, malignant cells frequently circumvent androgen dependence, progressing toward metastatic castration-resistant prostate cancer (CRPC). CRPC exhibits marked therapeutic resistance to conventional cytotoxic chemotherapy, secondary hormonal agents, and radiation therapy, while imposing significant clinical toxicities that impair patient functional status.³

Consequently, there is growing interest in screening plant-derived compounds and complementary therapeutic systems as potential supportive or adjunct modalities in oncology.⁴ Homeopathy, formulated by Samuel Hahnemann on the principle of *Similia Similibus Curentur*, utilizes serial succussion and ultra-high micro-dilutions to prepare medicinal remedies.³ Over the past two decades, preclinical evaluations have sought to determine whether homeopathic dilutions possess biological activity against malignant cell lines.⁴

Investigations into natural extracts and homeopathic preparations against prostate cancer cell lines (such as PC3, DU-145, and LNCaP) have documented varied bioactivities:

- MacLaughlin et al. reported that *Sabal serrulata* inhibited PC3 cell proliferation by 33% at 72 hours and DU-145 proliferation by 23% at 24 hours *in vitro*, with

significant reductions in prostate tumor xenograft dimensions *in vivo*.

- Jonas et al. and Thangapazham et al. observed that ultra-diluted centesimal potencies of *Thuja occidentalis*, *Conium maculatum*, and *Carcinosinum* produced negligible cytotoxicity and did not significantly alter mRNA levels of apoptotic regulators (*Bax*, *Bcl-2*, caspases) in selected prostate cancer models.
- Other natural formulations, such as *Echinacea angustifolia*, *Sorbus aria* (whitebeam) extracts, and *Tarantula cubensis* alcoholic extracts, have demonstrated concentration-dependent cytotoxic and apoptotic responses in *in vitro* oncological evaluations.⁵

Copaifera officinalis (commonly designated as *Copaiva officinalis* in homeopathic pharmacopoeias) is an oleoresin-bearing tree native to tropical South America.

Phytochemical analyses of *Copaifera* oleoresins reveal high concentrations of active sesquiterpenes (such as β -caryophyllene, α -copaene, and β -elemene) and diterpenic acids, which possess anti-inflammatory, antinociceptive, and antineoplastic properties.⁶ Isolated β -elemene has been shown to induce cell cycle arrest at the G2-M phase and activate autophagy-mediated apoptotic pathways in carcinoma models. In addition, *in vivo* animal studies have demonstrated the inhibitory activity of copaiba balsam on Walker 256 carcinoma⁵.

In homeopathic practice, *Copaiva officinalis* is utilized in concentrated Mother Tinctures (MT) and in serially dynamized dilutions, such as the 6C centesimal potency.⁵ However, empirical laboratory studies directly comparing the cytotoxic and anti-proliferative kinetics of the mother tincture versus dynamized potencies on human castration-resistant PC3 prostate cancer cells remain limited⁶

Therefore, the present study was designed to evaluate and compare the *in vitro* cytotoxic, metabolic-inhibitory, and anti-proliferative effects of *Copaiva officinalis* Mother Tincture and 6C potency on the PC3 prostate cancer cell line across 24, 48, and 72-hour exposure periods using MTT and Trypan blue exclusion assays⁶

2. MATERIALS AND METHODS

2.1 Test Preparations and Vehicle Controls

Copaiva officinalis Mother Tincture (MT) and *Copaiva officinalis* 6C potency were procured along with their respective vehicle controls (Dispensing Alcohol, DA). Reagents including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Trypan blue dye (0.4% w/v) were maintained under standard laboratory storage conditions.

2.2 Cell Culture

The androgen-independent PC3 human prostate cancer cell line was maintained in standard growth media supplemented with fetal bovine serum and antibiotics. Cultures were incubated in a humidified environment containing 5% CO₂ at 37°C.

2.3 MTT Colorimetric Viability Assay

1. **Cell Seeding:** PC3 cells were seeded into 96-well culture plates at a density of 1×10^4 cells/well and allowed to adhere for 24 hours.

2. **Treatment:** Cells were exposed to graded concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, and 64.0 $\mu\text{L/mL}$) of *Copaiva officinalis* MT, *Copaiva officinalis* 6C, or vehicle DA controls.
3. **Incubation:** Plates were incubated for 24, 48, or 72 hours in a 5% CO_2 incubator at 37°C .
4. **Reagent Addition:** The medium was refreshed with 100 μL of fresh medium, followed by the addition of 10 μL of MTT reagent (5 mg/mL in PBS) to each well.
5. **Formazan Development & Spectrophotometry:** Following a 4-hour incubation at 37°C , the generated formazan crystals were solubilized, and the absorbance (optical density, OD) was recorded at 540 nm using a multi-well microplate reader.

The percentages of cell viability and cytotoxicity were calculated using the following equations:

$$\text{Viability (\%)} = \left[\frac{\text{OD of treated cells}}{\text{OD of control cells}} \right] \times 100$$

$$\text{Cytotoxicity (\%)} = 100 - \text{Viability (\%)} = \left[\frac{\text{OD of control} - \text{OD of treated}}{\text{OD of control}} \right] \times 100$$

Half-maximal inhibitory concentration (IC_{50}) values were determined using concentration-response regression plots.

2.4 Trypan Blue Dye Exclusion Assay

1. **Harvesting:** Following treatment with MT, 6C, or vehicle DA for 24, 48, or 72 hours, PC3 cell suspensions were harvested and centrifuged.
2. **Resuspension & Staining:** The supernatant was discarded, and the cell pellet was resuspended in PBS. Trypan blue solution (0.4%) was added to the suspension at a 1:1 ratio and incubated at 25°C for 3 minutes.
3. **Microscopic Counting:** Cell suspensions were loaded into a hemocytometer and examined under bright-field microscopy. Live cells with intact membranes that excluded the dye (unstained) and dead cells with permeable membranes (stained blue) were enumerated to calculate cell growth and viability percentages.

3. RESULTS

3.1 MTT Cytotoxicity Profile of *Copaiva officinalis* Mother Tincture (MT)

Copaiva officinalis MT demonstrated strong concentration- and time-dependent cytotoxic activity against PC3 cells across all tested timepoints:

- **At 24 Hours:** Cytotoxicity increased from 29.01% at 0.5 $\mu\text{L/mL}$ to 79.47% at 64.0 $\mu\text{L/mL}$, compared to 1.94% and 55.42% in the DA
- **At 48 Hours:** Cytotoxicity ranged from 50.82% at 0.5 $\mu\text{L/mL}$ to 93.86% at 64.0 $\mu\text{L/mL}$, while DA control cytotoxicity was 6.76% and 67.06%, respectively.

Table 1: *In vitro* cytotoxicity (%) of *Copaiva officinalis* Mother Tincture (MT) and vehicle control (DA) on PC3 cells.

Concentration ($\mu\text{L/mL}$)	24 h MT (%)	48 h MT (%)	72 h MT (%)	Concentration ($\mu\text{L/mL}$)	24 h MT (%)	48 h MT (%)
0.5	29.01	50.82	54.32	0.5	29.01	50.82
1	52.40	56.30	61.25	1	52.40	56.30
2	57.96	69.30	77.26	2	57.96	69.30
4	61.50	80.37	89.31	4	61.50	80.37
8	70.73	89.35	90.99	8	70.73	89.35
16	76.01	91.80	92.15	16	76.01	91.80
32	77.16	93.56	97.57	32	77.16	93.56
64	79.47	93.86	97.58	64	79.47	93.86

At 72 Hours: Cytotoxicity reached 54.32% at 0.5 $\mu\text{L/mL}$ and peaked at 97.58% at 64.0 $\mu\text{L/mL}$

3.2 MTT Cytotoxicity Profile of *Copaiva officinalis* 6C Potency

The dynamized 6C preparation produced lower cytotoxic responses that required higher concentrations to manifest:

- **At 24 Hours:** Cytotoxicity was 2.76% at 0.5 $\mu\text{L/mL}$, rising to 43.08% at 16.0 $\mu\text{L/mL}$ and 57.15% at 64.0 $\mu\text{L/mL}$.
- **At 48 Hours:** Cytotoxicity remained low at lower concentrations (0.5 – 4.0 $\mu\text{L/mL}$), reaching 27.30% at 16.0 $\mu\text{L/mL}$ and 77.01% at 64.0 $\mu\text{L/mL}$.
- **At 72 Hours:** Cytotoxicity remained under 4% at doses up to 8.0 $\mu\text{L/mL}$, reaching 45.18% at 64.0 $\mu\text{L/mL}$.

Table 2: Cytotoxicity of *Copaiva officinalis* 6C in PC-3 cells

Concentration $\mu\text{L/mL}$	24h 6C (%)	48 h 6C (%)	48 h 6C (%)
0.5	2.76	9.79	-4.64
1	8.47	-7.35	-2.91
2	9.13	-5.03	-2.38
4	20.17	-4.60	0.24
8	36.19	3.56	3.03
16	43.08	27.30	16.73
32	53.45	56.70	26.88
64	57.15	77.01	45.18

3.3 Comparison of Half-Maximal Inhibitory Concentrations (IC₅₀)

Calculated IC₅₀ metrics confirmed that the Mother Tincture possessed high potency relative to its corresponding vehicle control and the 6C ultra-dilution

- **Mother Tincture:** Exhibited an IC₅₀ of 1.41 $\mu\text{L/mL}$ at 24 hours, which decreased to 0.595 $\mu\text{L/mL}$ at 48 hours and 0.45 $\mu\text{L/mL}$ at 72 hours
- **6C Potency:** Exhibited an IC₅₀ of 28.85 $\mu\text{L/mL}$ at 24 hours, 41.55 $\mu\text{L/mL}$ at 48 hours, and 42.95 $\mu\text{L/mL}$ at 72 hours.

Table 3: Half-maximal inhibitory concentration (IC₅₀) values of *Copaiva officinalis* formulations and vehicle controls on PC3 cells.

Treatment	24 h ($\mu\text{L/mL}$)	48 h ($\mu\text{L/mL}$)	72 h ($\mu\text{L/mL}$)
<i>Copaiva officinalis</i> MT	1.41	0.595	0.45
<i>Copaiva officinalis</i> 6C	28.85	41.55	42.95
Control DA for MT	38.65	34.60	30.05
Control DA for 6C	47.05	58.00	81.00

3.4 Trypan Blue Cell Proliferation Kinetics

The Trypan blue exclusion assay confirmed that *Copaiva officinalis* MT produced marked inhibition of PC3 cell proliferation relative to 6C potency and control groups across all timepoints:

- **24-Hour Incubation:** Viable cell recovery was 16.95% for MT (vs. 59.32% for DA control) and 63.56% for 6C potency (vs. 75.42% for DA control).
- **48-Hour Incubation:** Viable cell recovery was 14.29% for MT (vs. 55.78% for DA control) and 46.94% for 6C potency (vs. 82.99% for DA control).
- **72-Hour Incubation:** Viable cell recovery decreased to 4.76% for MT (vs. 47.62% for DA control), whereas 6C maintained 78.57% viability (vs. 79.76% for DA control).

Table 4: Trypan blue cell viability (%) across incubation periods.

Treatment Group	IC ₅₀ at 24 h (μL/mL)	IC ₅₀ at 48 h (μL/mL)	IC ₅₀ at 72 h (μL/mL)	Treatment Group	IC ₅₀ at 24 h (μL/mL)
<i>Copaiva officinalis</i> (MT)	1.41	0.595	0.45	<i>Copaiva officinalis</i> (MT)	1.41
Control DA for MT	38.65	34.60	30.05	Control DA for MT	38.65
<i>Copaiva officinalis</i> (6C)	28.85	41.55	42.95	<i>Copaiva officinalis</i> (6C)	28.85
Control DA for 6C	47.05	58.00	81.00	Control DA for 6C	47.05

4. DISCUSSION

Castration-resistant prostate cancer (CRPC) presents substantial therapeutic challenges due to intrinsic and acquired resistance mechanisms against standard chemotherapeutic and hormonal agents. Identifying plant-derived natural compounds and evaluating integrative therapeutic options may provide insights into supportive cancer care.

In the present study, *Copaiva officinalis* Mother Tincture displayed high cytotoxic and anti-proliferative activities against human androgen-independent PC3 prostate cancer cells. The reduction in cell viability (> 97% cytotoxicity at 64.0 μL/mL) and the low IC₅₀ value (0.45 μL/mL at 72 h) demonstrate strong dose-dependent efficacy. These results align with previous phytochemical investigations of *Copaifera* oleoresins, which identified bioactive sesquiterpenes (such as β-caryophyllene, α-copaene, and β-elemene) and diterpenoids known to disrupt cell membrane integrity, inhibit cell cycle progression, and initiate apoptosis.

In comparison, the dynamized centesimal preparation *Copaiva officinalis* 6C exhibited lower direct cytotoxicity, with an IC₅₀ of 28.85 μL/mL at 24 hours, and showed viability levels closer to the vehicle controls at 48 and 72 hours. This difference between crude mother tinctures and potentized high dilutions is consistent with observations in the literature:

- Studies on *Sabal serrulata* and *Echinacea angustifolia* have reported direct growth inhibition or apoptosis induction in specific cell line models.
- Conversely, evaluations of high dilutions of *Thuja occidentalis*, *Conium maculatum*, and *Carcinosinum* by Jonas et al. and Thangapazham et al. demonstrated no

significant direct cytotoxic effects or changes in apoptotic gene expression (*Bax*, *Bcl-2*, *Fas*, *caspases*) in prostate cancer lines *in vitro*.

These findings indicate that mother tinctures act primarily through direct biochemical interactions mediated by their constituent phytochemicals, whereas ultra-diluted dynamized potencies may operate through alternative regulatory or non-cytotoxic pathways. Additional research utilizing flow cytometry (Annexin V/PI staining) and molecular profiling of apoptotic markers (caspase-3, PARP cleavage, *Bax/Bcl-2*) will help further elucidate the mechanistic pathways involved.

5. CONCLUSION

Copaiva officinalis demonstrates distinct, concentration- and time-dependent *in vitro* cytotoxic and anti-proliferative effects on human PC3 prostate cancer cells. The Mother Tincture formulation showed strong cytotoxic activity, significantly reducing viable cell numbers with an IC₅₀ of 0.45 μ L/mL at 72 hours. The ultra-diluted 6C potency exhibited moderate anti-proliferative effects, differing distinctly from the mother tincture. These results provide preliminary preclinical evidence supporting further mechanistic and molecular evaluation of *Copaiva officinalis* preparations in oncological research.

DECLARATIONS

- **Funding:** This research received financial support under institutional research sanction.
- **Conflict of Interest:** The authors declare that they have no competing financial or non-financial interests.
- **Author contributions:**

Dr. Monali T. Thopte: Conceptualization of the study, supervision, study design, interpretation of findings, manuscript review and critical revision, and final approval of the manuscript.

Dr. Aniket Mali: Experimental work, cell culture and laboratory investigation, MTT and Trypan Blue assays, data collection and analysis, interpretation of experimental results

Dr Anita Patil: Dean & I/C Principal Bharati Vidyapeeth (Deemed to be University), Homoeopathic Medical College, Pune- Institutional coordination, administrative facilitation of university funding, provisioning of core laboratory resources, and project oversight.

Dr. Priya Seth: Literature review, manuscript preparation, critical review, editing, and revision of the final manuscript.

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