

Evaluating Anticancer Activity of *Karveeradi Yoga* in Cervical Cancer Cell Lines: An In-vitro Study Research Protocol

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ABSTRACT

Introduction: Ayurveda, a traditional Indian system of medicine, emphasises the use of natural remedies. *Karveeradi Yoga*, derived from classical Ayurvedic scriptures, consists of potent herbs such as *Nerium indicum* and *Moringa oleifera*, which have traditionally been used for detoxification and for treating conditions resembling cancer. Cervical cancer, a significant global health burden, arises from persistent Human Papillomavirus (HPV) infections and necessitates the exploration of effective alternative treatment options.

Need of the study: The rising incidence of cervical cancer highlights the limitations of conventional therapies, including their high toxicity, cost, and adverse side-effects. In view of these challenges, exploring the anticancer potential of Ayurvedic formulations such as *Karveeradi Yoga* may offer safer and more affordable therapeutic alternatives.

Aim: To standardise *Karveeradi Yoga* and evaluate its anticancer activity in cervical cancer cell lines.

Materials and Methods: An in-vitro experimental preclinical study will be conducted from June 2025 to December 2025 at the Biocyte Institute of Research and Development, Sangli, Maharashtra India, in collaboration with the Department of Agada Tantra, Mahatma Gandhi Ayurved College and Hospital and Research Centre, Wardha, Maharashtra, India. Raw materials will be authenticated, standardised, and prepared according to *Bhavprakash Samhita*. Physicochemical and phytochemical analyses will be performed. Two cervical cancer cell lines, HeLa and SiHa, will be subjected to MTT assay, flow cytometry, and IC₅₀ determination. Positive controls (standard chemotherapy agents) and vehicle controls will be included. Statistical analysis will be performed using Analysis of Variance (ANOVA) or t-tests, with p-value <0.05 considered statistically significant.

Keywords: Apoptosis, Cervical neoplasms, Flow cytometry, Plant preparations, Phytotherapy

INTRODUCTION

Ayurveda has ancient roots, originating in India, and is considered one of the oldest holistic healing systems in the world. Its comprehensive approach and emphasis on natural remedies have gained global recognition. One of the eight branches of Ayurveda, *Agada Tantra*, was highly developed in antiquity and remains essential for detoxification therapies. Classical texts provide detailed descriptions of the classification, evaluation, and treatment of poisons (*Visha*), which are substances harmful to health when consumed, inhaled, or absorbed.

Poisons (*Visha*) are classified into two categories: *Jangam* (animal-derived poisons such as those from snakes, rats, and spiders) and *Sthavar* (plant-derived poisons such as *Dhatura* and *Vatsanabh*). Ancient Acharyas stated that *Agada* formulations neutralise various toxic substances, thereby counteracting the effects of *Visha*. An additional therapeutic application of *Agada Tantra* is *Visha Kalpa*, which refers to formulations containing poisonous substances used therapeutically to treat diseases [1].

In contemporary life, individuals are regularly exposed to multiple toxins, which accumulate over time as *Dooshivisha* (cumulative poisoning) [2]. According to the GLOBOCAN 2022 fact sheet, cervical cancer is the fourth most common cancer in women worldwide, with an estimated 662,301 new cases (age-standardised rate [ASR]: 14.1 per 100,000), and the ninth leading cause of cancer-related deaths, accounting for 348,874 deaths (ASR: 7.1 per 100,000). The global five-year prevalence is approximately 1.95 million cases. Asia accounts for nearly 60% of cases and 57% of deaths, followed by Africa and Latin America. The disease burden is unevenly distributed, with countries such as Eswatini reporting the highest incidence and mortality rates (incidence: 95.9; mortality: 64.3 per 100,000) [3].

Women diagnosed with cervical cancer often experience a substantial decline in overall quality of life. Despite advances in treatment modalities such as surgery, chemotherapy, and radiotherapy,

median survival in advanced metastatic stages remains between 8 and 13 months [4].

In Ayurveda, *Arbuda* (benign or malignant tumour) is described as a round (*vritta*), immobile (*achala*), mildly painful (*mridu-ruja*), and deep-rooted (*gambhira-mula*) mass that grows continuously (*chiranubandhi*) and does not regress spontaneously [5]. This description closely aligns with the modern medical definition of a solid malignant tumour [6].

According to Ayurvedic pathology, aggravated *Vata* is responsible for abnormal cell proliferation and rapid tumour growth, leading to disruption of bodily systems and structures. *Pitta* is associated with fever and abnormal bleeding observed in *Arbuda*, while aggravated *Kapha* primarily contributes to uncontrolled increases in cell mass [7]. Classical Ayurvedic texts emphasise purification, dosha balance, and immune strengthening through herbal formulations for the management of *Arbuda*.

Acharya *Bhavprakash* described a formulation known as *Karveeradi Yoga*, in which *Karveer* (*Nerium indicum* Mill.) root, *Sarshap* (*Brassica juncea* [Linn.] Czern.) seed, *Sursa* (*Ocimum sanctum* Linn.) leaves, *Indrayava* (*Holarrhena antidysenterica* [Linn.] Wall.) seed, *Mulak* (*Raphanus sativus* L.) seed, and *Shigru* (*Moringa oleifera* Lam.) seed are coarsely powdered and mixed with buttermilk (*Takra*) [8]. This formulation is believed to support the body's natural healing mechanisms and promote the breakdown of aberrant tissue growth. Nevertheless, the use of these potent herbs may exert significant therapeutic effects.

REVIEW OF LITERATURE

Karveeradi Yoga is a classical Ayurvedic formulation comprising six ingredients: *Moringa oleifera*, *Raphanus sativus* (radish), *Brassica juncea* (mustard), *Ocimum sanctum* (basil), *Holarrhena antidysenterica* seeds, and *Nerium indicum*. It is traditionally indicated for the management of benign and malignant tumours.

Contemporary pharmacological studies have demonstrated in-vitro anticancer activity for each of these individual components, thereby supporting the rationale for evaluating this compound formulation against cancer cell lines.

Karveera (*Nerium indicum*) contains the cardiac glycoside oleandrin, which is responsible for its therapeutic effects. By inhibiting Na⁺/K⁺-ATPase, oleandrin increases intracellular Na⁺ and Ca²⁺ levels, resulting in positive inotropy and arrhythmogenic toxicity [9]. In addition to modulating the NF-κB, MAPK, PI3K/Akt, and STAT3 signalling pathways, oleandrin induces cell-cycle arrest, apoptosis, Reactive Oxygen Species (ROS) generation, mitochondrial dysfunction, and radiosensitisation [10].

Experimental studies have demonstrated its anticancer effects. *N. oleander* extracts inhibited HeLa cell proliferation by inducing G2/M arrest and suppressing EGFR/pRb signalling [11]. Furthermore, oleandrin triggered immunogenic cell death characterised by Calreticulin (CRT) exposure, HMGB1 release, and ATP secretion, thereby enhancing antitumour immunity in-vivo [12].

Despite these promising effects, significant safety concerns remain. Human poisoning is associated with arrhythmias, neurotoxicity, and multiorgan failure, while animal studies have demonstrated dose-dependent hepatotoxicity, including degeneration, haemorrhage, and apoptosis [13]. Nevertheless, standardised formulations such as PBI-05204 and Anvirzel® are currently under clinical evaluation for antiviral and anticancer applications [14].

Nerium indicum (*Karveera*), despite its recognised toxicity, is a classical component of *Karveeradi Yoga*. In this formulation, traditional processing methods and polyherbal synergy are believed to mitigate adverse effects while preserving therapeutic potential [11].

Moringa oleifera seed extracts have demonstrated a broad spectrum of anticancer effects in-vitro. Sugar-rich and crude extracts inhibited migration, clonogenicity, and proliferation in breast cancer cell lines (MCF-7 and MDA-MB-231) [15]. Purified MIC-1 (moringin) inhibited renal cancer cell growth via modulation of the PTP1B–Src/Ras/Raf/ERK signalling pathway [16].

Radish (*Raphanus sativus*) seeds are rich in glucoraphenin and its isothiocyanate derivative, Sulforaphene (SFE). Sulforaphene has been shown to selectively inhibit breast cancer cell proliferation at concentrations of 5–10 μM by inducing G2/M cell-cycle arrest, promoting apoptosis through oxidative stress and mitochondrial dysfunction, and activating cytoprotective autophagy [17]. Broad-spectrum anticancer effects have also been reported in colon (HT-29, HCT116), cervical (HeLa), and prostate (LNCaP) cancer cell lines, including PARP cleavage and caspase activation [18].

Mustard seed powder (MSP-1; 71.5 mg/kg; equivalent to 9 μmol/kg sinigrin) completely prevented muscle invasion (100%) and inhibited bladder tumour growth by 34.5% (p-value <0.05) in-vivo. These effects were associated with modulation of VEGF, cyclin B1, and caspase-3 expression. As a natural anticancer delivery system, MSP-1 demonstrated superior efficacy compared to pure allyl isothiocyanate (AITC) [19].

Ursolic acid (UA), isolated from basil leaves, suppresses NF-κB, STAT3, β-catenin, and EGFR signalling, thereby reducing colorectal cancer growth and angiogenesis in mouse models. Additionally, UA enhances the therapeutic efficacy of capecitabine through synergistic interactions [20].

Holarrhena antidysenterica (*Indrayava*) contains steroidal alkaloids, including conessine. Leaf extracts have demonstrated strong cytotoxic activity against 14 human cancer cell lines in-vitro. Among the fractions tested, the chloroform fraction exhibited maximal activity. Ethanolic extracts were active against lung, colon, liver, oral, ovarian, cervical, and neural cancers, while hydroalcoholic extracts showed activity against most cancer types except ovarian cancer. Aqueous extracts achieved greater than 50% inhibition in lung and colon cancer cell lines [21].

The present study evaluates *Karveeradi Yoga* as a standardised whole formulation in cancer cell lines, incorporating phytochemical profiling, comparison with standard chemotherapeutic agents, and parallel toxicity testing on normal cells to assess both efficacy and safety. This approach aims to provide preclinical insights into the formulation's translational potential.

Objectives:

- To prepare *Karveeradi Yoga* and study its physicochemical and phytochemical properties using High-Performance Liquid Chromatography (HPLC).
- To evaluate the anticancer activity of *Karveeradi Yoga* in two cervical cancer cell lines (HeLa and SiHa) by assessing its effects on cell viability, proliferation, and cytotoxicity using MTT assay and flow cytometry.

Null hypothesis: *Karveeradi Yoga* does not exhibit significant anticancer activity against cervical cancer cell lines (HeLa and SiHa).

Alternative hypothesis: *Karveeradi Yoga* exhibits significant anticancer activity against cervical cancer cell lines (HeLa and SiHa).

MATERIALS AND METHODS

This experimental preclinical in-vitro cell line study will be conducted at Mahatma Gandhi Ayurved College, Hospital, and Research Centre, Salod (H), Wardha, Maharashtra, and the Biocyte Institute of Research and Development, Sangli, Maharashtra, India over a period of six months (June 2025–December 2025). The study has been approved by the Institutional Ethics Committee with reference number MGACHRC/IEC/Jun-2024/831. As this is an in-vitro experimental study without human or animal participants, informed consent is not applicable.

Inclusion criteria:

- HeLa (adenocarcinoma) and SiHa (squamous cell carcinoma) human cervical cancer cell lines.
- At least one normal/healthy human-derived cell line (e.g., HaCaT keratinocytes, HEK293, or equivalent) for comparative cytotoxicity analysis.
- Cell lines authenticated by Short Tandem Repeat (STR) profiling or an equivalent method within the last six months.
- Mycoplasma-free and free from other microbial contamination.
- Proven viability ≥90% at study initiation.
- Cell lines and test compounds directly relevant to cervical cancer research and cytotoxicity evaluation.

Exclusion criteria:

- Cell lines contaminated with mycoplasma, bacteria, fungi, or cross-contaminated with other cell lines.
- Cell lines not validated/authenticated for the intended study purpose.
- Raw materials or drugs that fail API standard tests or lack a certificate of analysis.
- Any cell lines exhibiting genetic or phenotypic drift that compromise study validity.

Preparation of *Karveeradi Yoga*: The ingredients required to prepare *Karveeradi Yoga*—*Karveer* (*Nerium indicum* Mill.), *Sarshap* (*Brassica juncea* (Linn.) Czern.), *Sursa* (*Ocimum sanctum* Linn.), *Indrayava* (*Holarrhena antidysenterica* (Linn.) Wall.), and the seeds of *Mulak* (*Raphanus sativus* L.) and *Shigru* (*Moringa oleifera* Lam.) [Table/Fig-1]—will be procured from Wagh Brothers, Nagpur, Maharashtra, a vendor cultivating and supplying medicinal herbs according to good manufacturing practices [22–27].

The Department of Dravyaguna, Mahatma Gandhi Ayurvedic College, Hospital, and Research Centre, Salod, Wardha, Maharashtra, will validate these drugs based on their morphological and organoleptic characteristics.

S. No.	Name of Drug	Botanical name	Family	Part used	Quantity
1.	<i>Karveer</i> [22]	<i>Nerium indicum</i> Mill.	Apocynaceae	Mula	500 gm
2.	<i>Sarshap</i> [23]	<i>Brassica juncea</i> Linn.	Cruciferae	Beeja	500 gm
3.	<i>Surasa</i> [24]	<i>Ocimum sanctum</i> Linn	Lamiaceae	Patra	500 gm
4.	<i>Indrayava</i> [25]	<i>Holarrhena antidysenterica</i> Linn	Apocynaceae	Beeja	500 gm
5.	<i>Shigru beeja</i> [26]	<i>Moringa oleifera</i> Lam.	Moringaceae	Beeja	500 gm
6.	<i>Mulak beeja</i> [27]	<i>Raphanus sativus</i> L	Brassicaceae	Beeja	500 gm

[Table/Fig-1]: *Karveeradi Yoga* components [22-27].

To prepare *Karveeradi Yoga*, the seeds of *Shigru*, *Mulak*, *Sarshap*, and *Indrayava*, along with the leaves of *Sursa* and *Karveer*, will be collected in equal quantities, sun-dried, and then ground, crushed, or chopped into a fine powder (*churna*). This powder will be thoroughly mixed and combined with a sufficient quantity of buttermilk (*Takra*) [8,28]. To ensure uniformity and effectiveness, the formulation will be standardised according to the Ayurvedic Pharmacopoeia of India (API) criteria [29].

Cell line sample: HeLa and SiHa human cervical cancer cell lines will be procured from the Biocyte Institute of Research and Development, Sangli, Maharashtra, India, which will also serve as the site for conducting the experimental study.

Cancer Cell Line Culture [30]

The selected cancer cell lines will undergo quality control inspection before being cryopreserved in liquid nitrogen vapours using DMSO (5-10%). Cells will be cultured in RPMI 1640 medium in cell culture flasks. For growth, the flasks will be maintained in a CO₂ incubator at 37°C for 24-48 hours. Cell counting will be performed using a haemocytometer (Neubauer Improved, Marienfeld Superior, Germany), and the cell density adjusted based on titration readings (approximately 1×10⁵ cells/mL). All cell culture work will be conducted under strictly aseptic conditions inside a laminar flow hood to prevent bacterial contamination.

Preparation of Solution of Test Drug

The test drug stock solution was prepared at a concentration of 64 mg/mL in ethanol. A working stock solution of 1600 µg/mL was prepared by diluting 25 µL of the stock solution with 975 µL of complete medium [31]. Further dilutions were carried out to obtain final test concentrations of 40, 80, 120, and 160 µg/mL by adding 25 µL, 50 µL, 75 µL, and 100 µL of the working stock to 975 µL, 950 µL, 925 µL, and 900 µL of complete medium, respectively. All solutions were prepared freshly before use, and ethanol concentration was kept constant in all experimental groups [30].

Plate Preparation and Drug Addition

Cells will be seeded at 100 µL per well onto 96-well microtiter plates, yielding approximately 5×10³ cells per well. Plates will be incubated for 24 hours at 37°C in 5% CO₂, 95% air, and 100% relative humidity. To maintain aseptis, drug addition will be performed under a laminar flow hood. Cells will then be treated with *Karveeradi Yoga* at various concentrations.

OUTCOMES

Analytical Study Parameter for *Karveeradi Yoga*

1. Organoleptic characterisation:

- Visual attributes: colour, surface texture, morphology
- Olfactory profile: characteristic aroma
- Gustatory analysis: palatability assessment
- Tactile features: consistency, granularity

2. Physicochemical profiling:

- Moisture content determination (Loss on Drying at 105°C)

- Total ash value estimation
- Acid-insoluble ash measurement
- pH determination (1% and 10% aqueous solutions)
- Water-soluble extractive value
- Alcohol-soluble extractive value
- Particle size distribution analysis
- Microbial load assessment (total viable count, yeast and mold count, pathogen detection)

3. Phytochemical profiling:

- **Chromatographic fingerprinting:** HPTLC profiling for marker compounds
- **Qualitative phytochemical screening:**
 - Alkaloid identification
 - Carbohydrate and glycoside detection
 - Saponin screening
 - Protein and free amino acid detection
 - Volatile oil estimation

Cell morphology and behavioural responses will be observed using subjective assessment parameters [Table/Fig-2], while cell viability, cell-cycle analysis, cytotoxicity, and apoptosis will be evaluated using objective parameters [Table/Fig-3].

Cell morphology observation	Visual inspection of HeLa and SiHa cervical cancer cell lines will be carried out using a phase-contrast microscope (Magnification: 40× for general morphology, 100× for detailed nuclear changes). Observations will focus on: Cell shape (rounding, shrinking, or swelling). Detachment from the culture plate. Signs of apoptosis (e.g., blebbing, chromatin condensation). Necrotic features (e.g., loss of membrane integrity).
Behaviour of cancer cells	Response to drug concentrations, including: Decreased proliferation. Changes in adhesion properties.
Drug precipitation or interaction	Observing clarity and consistency of the <i>Karveeradi Yoga</i> in the wells. Ensuring no interference due to precipitation of the <i>Karveeradi Yoga</i> .

[Table/Fig-2]: Subjective criteria.

Cell growth and viability	Reduction in percentage viability will be taken as an indicator of the cytotoxic effect of <i>Karveeradi Yoga</i> . Cell viability, growth, and proliferation will be evaluated in relation to various environments and therapeutic concentrations using a colorimetric MTT assay. Procedure: After drug treatment for specified incubation periods (24 h, 48 h, 72 h), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution will be added to each well. Metabolically active cells will reduce MTT to formazan crystals, which will be dissolved in DMSO, and optical density will be measured at 570 nm using a microplate reader. Analysis: % cell viability will be calculated relative to untreated controls, and growth curves will be plotted against drug concentration.
Cell cycle analysis	Cell cycle distribution will be analysed by flow cytometry (FACS) after propidium iodide (PI) staining. Cells will be harvested post-treatment, fixed in 70% ethanol, and stained with PI in the presence of RNase A. Flow cytometric analysis will determine the proportion of cells in G0/G1, S, and G2/M phases. Arrest at specific phases (e.g., G0/G1) will be interpreted as an indication of proliferation inhibition.

Measuring cytotoxicity	Cytotoxicity will be quantified as IC ₅₀ , the concentration of <i>Karveeradi Yoga</i> that reduces cell viability by 50%: Viability data from the MTT assay will be plotted against drug concentration. Non-linear regression analysis (sigmoidal dose-response curve) will be performed using software such as GraphPad Prism to calculate IC ₅₀ values for HeLa and SiHa cells at different incubation times.
Apoptosis determination	The mode of cell death will be determined by Annexin V-FITC/Propidium Iodide dual staining followed by flow cytometry [32]: Annexin V-FITC positive/PI negative cells will be classified as early apoptotic. Annexin V-FITC positive/PI positive cells will be classified as late apoptotic or secondary necrotic. Annexin V-FITC negative/PI positive cells will be classified as necrotic. The ratio of apoptotic to necrotic cells will be compared between treated and control groups.

[Table/Fig-3]: Objective criteria [32].

STATISTICAL ANALYSIS

Data will be analysed using Statistical Package for the Social Sciences (SPSS) Statistics (version 19.0). Statistical comparisons will be performed using two-way ANOVA and an unpaired, two-tailed Student's t-test. Statistical significance will be set at p-value <0.05. All results will be presented as mean±Standard Deviation (SD) from at least three independent experiments.

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