

Phytochemical Profiling and Anticancer Evaluation of a Polyherbal Extraction reaction Caco-2 Cell Line Using the MTT Assay

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ABSTRACT

The increasing prevalence of cancer and the limitations of conventional therapies have accelerated the search for safer, cost-effective, and naturally derived anticancer agents. Polyherbal formulations, prepared by combining bioactive medicinal plants, offer synergistic therapeutic potential with reduced toxicity and enhanced pharmacological activity. The present study focuses on the formulation and evaluation of a polyherbal decoction prepared from *Hibiscus rosa-sinensis* petals, *Azadirachta indica* (Neem) leaves, and *Carica papaya* leaves. These plants were selected based on their traditional medicinal importance and reported anticancer, antioxidant, and immunomodulatory properties. The powders of each plant material were subjected to decoction extraction using distilled water as solvent to obtain a concentrated aqueous extract. The extract was evaluated for its physicochemical properties, including total ash, acid-insoluble ash, water-soluble ash, and moisture content, ensuring purity and quality. Preliminary phytochemical screening confirmed the presence of flavonoids, alkaloids, tannins, steroids, carbohydrates, and glycosides, which are known to contribute to anticancer activity. The polyherbal extract was then tested for its cytotoxic potential using the Caco-2 cancer cell line through the MTT assay. This colorimetric test measures cell viability based on mitochondrial dehydrogenase activity. The results demonstrated a significant, dose-dependent reduction in cell viability, indicating strong cytotoxic and growth-inhibitory effects of the extract against cancer cells. The findings suggest that the formulated polyherbal decoction possesses promising anticancer potential, possibly due to synergistic interactions of phytoconstituents from *Hibiscus*, *Neem*, and *Papaya*. This study supports the potential use of polyherbal extracts as natural therapeutic agents in cancer management and highlights the importance of integrating traditional herbal knowledge with modern cytotoxicity assays for drug discovery.

Keywords: Polyherbal Decoction, Anticancer Activity, *Hibiscus rosa-sinensis*, *Azadirachta indica*, *Carica papaya*.

INTRODUCTION

Cancer is one of the most severe and complex diseases that continues to threaten human life globally. It is characterized by the uncontrolled proliferation of abnormal cells that have the potential to invade and destroy surrounding tissues and spread to distant organs through metastasis. Despite tremendous advances in medical science, cancer remains one of the leading causes of mortality worldwide. Modern therapeutic approaches such as chemotherapy, radiotherapy, and immunotherapy have undoubtedly improved survival rates; however, they are often accompanied by severe adverse effects including

damage to normal cells, development of drug resistance, and a high recurrence rate. Moreover, the high cost of treatment and the limited availability of modern therapies in developing regions create an urgent need for safer, more accessible, and affordable alternatives. This growing concern has shifted global attention toward natural sources, particularly medicinal plants, as potential reservoirs of bioactive compounds capable of combating cancer through multiple mechanisms with minimal toxicity.

Medicinal plants have been used for centuries as an integral part of traditional healing systems such as Ayurveda, Siddha, and Chinese medicine. These

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systems emphasize the holistic use of plant-based formulations for the prevention and treatment of diseases. Unlike synthetic drugs that usually act on a single molecular target, herbal preparations contain a wide range of phytoconstituents that act synergistically to provide therapeutic benefits. Such polyherbal combinations are especially valuable because they not only enhance pharmacological efficacy but also reduce side effects and toxicity. The complexity of cancer demands multi-targeted approaches, and herbal medicines are inherently capable of addressing this need through their composite phytochemical profiles. Phytochemicals such as flavonoids, alkaloids, terpenoids, tannins, saponins, and phenolic compounds exhibit antioxidant, anti-inflammatory, and cytotoxic properties that are essential in counteracting carcinogenesis. These compounds modulate key molecular pathways associated with cell cycle regulation, apoptosis, and oxidative stress, which are directly involved in cancer development and progression. In recent years, the exploration of traditional medicinal plants for anticancer activity has gained remarkable scientific interest. Among the numerous plants known for their therapeutic potential, *Hibiscus rosa-sinensis* (Hibiscus flower), *Azadirachta indica* (Neem), and *Carica papaya* (Papaya) stand out for their broad pharmacological activities and ethnomedicinal relevance. The flowers of *Hibiscus rosa-sinensis* are rich in anthocyanins, flavonoids, and phenolic compounds known for their potent antioxidant and cytotoxic effects. These bioactive molecules neutralize free radicals and reactive oxygen species, thereby preventing oxidative DNA damage a crucial factor in cancer initiation. Studies have demonstrated that *Hibiscus* extracts can inhibit cancer cell proliferation, induce apoptosis, and suppress tumor-promoting enzymes. Similarly, *Azadirachta indica*, commonly known as Neem, is revered in Ayurvedic medicine as a universal healer due to its wide spectrum of biological activities. Neem leaves contain limonoids, nimbin, quercetin, and azadirachtin, compounds that exhibit immunomodulatory, anti-inflammatory, and antiproliferative effects. Neem extracts have been found to interfere with cancer cell signaling pathways, inhibit angiogenesis, and induce programmed cell death in various cancer cell lines. *Carica papaya*, another highly valued medicinal plant, possesses

strong pharmacological activities attributed to its rich composition of alkaloids, flavonoids, papain enzyme, and phenolic acids. The leaf extract of papaya has been reported to demonstrate cytotoxic activity against several types of cancer cells, as well as immune-enhancing properties through the stimulation of cytokine production and activation of natural killer cells. The rationale behind combining these three plants in a single polyherbal decoction lies in their complementary and synergistic mechanisms of action. While *Hibiscus* contributes powerful antioxidants that prevent oxidative stress and DNA mutation, *Neem* provides potent immunomodulatory and antiproliferative effects, and *Papaya* supports enzymatic detoxification, immune activation, and apoptosis induction. Together, these plants form a well-balanced combination capable of targeting multiple hallmarks of cancer, including oxidative stress imbalance, inflammation, abnormal cell proliferation, and impaired immune surveillance. The holistic nature of this formulation reflects the principles of traditional medicine, which emphasize the combined effect of several herbs to achieve enhanced therapeutic benefits compared to single plant extracts. The present research is therefore aimed at the preparation of a polyherbal aqueous decoction using *Hibiscus rosa-sinensis*, *Azadirachta indica*, and *Carica papaya* as key plant materials, followed by its preliminary evaluation for anticancer potential. The study focuses primarily on the extraction process using the decoction method, ensuring that the formulation remains simple, reproducible, and close to traditional practices. The scientific documentation of this process is essential to bridge traditional knowledge with modern pharmacological understanding, thereby contributing to the growing field of evidence-based herbal medicine. Through this study, it is anticipated that the polyherbal decoction will demonstrate significant potential as a safe, natural, and cost-effective anticancer formulation, encouraging further analytical, biological, and clinical investigations in the future[2,3,4,5,6,9,13,14,18]

2. MATERIALS & METHODOLOGY:

2.1. Material:

Fresh *Hibiscus* flowers, *Neem* leaves, and *Papaya* leaves were collected from local botanical sources and shade dried for 10 days. The dried plant materials



were ground into fine powder using a mechanical grinder and stored in airtight containers for extraction. Analytical grade distilled water was used as the extraction solvent. All glassware used was cleaned and sterilized before use.

2.2. Chemical and Reagents:

Hydrochloric Acid, Mayer's Reagent, Magnesium Turnings, Ferric Chloride Solution, Acetic Acid, Concentrated Sulphuric Acid, Alpha-Naphthol Solution

2.3. Preparation of Herbal Extract:

The decoction extraction method was adopted for the preparation of polyherbal extract. Equal quantities (50 g each) of *Hibiscus rosa-sinensis* flower powder, *Azadirachta indica* leaf powder, and *Carica papaya*

leaf powder were mixed thoroughly. The combined 150 g of powdered material was transferred to a clean beaker containing 1000 mL of distilled water. The mixture was boiled gently on a heating mantle for 45 minutes until the volume was reduced to one-third of its original volume 350 mL. The decoction was allowed to cool at room temperature, filtered through Whatman No. 1 filter paper, and the filtrate was collected. The obtained aqueous extract was concentrated on a water bath at 60 °C to yield a thick extract. The final extract was stored in sterile airtight containers at 4 °C for further phytochemical and biological evaluation[8,16].

3. QUALITATIVE ANALYSIS:

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able No.1. Phytochemical Screening by Chemical Test[7,10,11,15].

TEST	PROCEDURE
Alkaloids (Mayer's Test)	Take a test tube add 2ml extract sample + few drops HCl and heat solution + Mayer's reagent, white precipitate observed
Flavonoids (Shinoda Test)	Take a test tube add 2ml extract sample+ 0.5gmMagnesium Turning + Few drops of Con ^c HCl Pink colour will observe.
Glycosides (Keller Kiliani Test)	Take a test tube add 2 ml extract + 1mlacetic acid+FeCl ₃ + few drops Con ^c H ₂ SO ₄ Brown Colour will observe.
Carbohydrates (Molisch's Test)	Take a test tube add 2ml extract + Alpha Naphthol Solution + Con ^c H ₂ SO ₄ observed violet zone or layer will observe.
Steroids (Liebermann-Burchard)	Take a test tube add 1ml extract+ 2 ml acetic acid+ Con ^c H ₂ SO ₄ Observed violet colour.
Tannins (FeCl₃ Test)	Take a test tube add 2ml extract+ FeCl ₃ solution will Observed deep blue and black colour.

4. QUANTITATIVE ANALYSIS

4.1. Total Ash:

Fifteen grams of powdered plant materials of *Hibiscus rosa-sinensis* petals, *Azadirachta indica* leaves, and *Carica papaya* leaves were accurately weighed and placed in a silica crucible. The samples were incinerated in a muffle furnace at 550 °C for 60 minutes until grayish ash remained, indicating complete removal of organic matter. The total ash values reflected the natural mineral content without significant contamination, with Neem leaves showing the highest ash content[12].

4.2. Acid-Insoluble Ash:

The total ash obtained from the muffle furnace was boiled with dilute hydrochloric acid, and the insoluble fraction was collected, dried, and weighed. Low acid-insoluble ash values were observed in all samples, indicating minimal siliceous contamination. Hibiscus petals showed the lowest residue, while Papaya leaves had slightly higher content, likely due to minor surface soil adherence[4].

4.3. Water-Soluble Ash:

The total ash was boiled with distilled water, and the insoluble portion was removed. The water-soluble ash fraction was highest in Hibiscus petals, reflecting a substantial proportion of water-soluble minerals. All samples showed consistent mineral solubility suitable for decoction preparation and extraction of bioactive constituents[12].



4.4. Moisture Content (Loss on Drying):

Known quantities of the powdered samples were dried in an oven at 105 °C until constant weight. Moisture content was within acceptable limits, ensuring stability, preventing microbial growth, and confirming suitability for decoction preparation. Neem leaves contained slightly higher moisture compared to Hibiscus petals and Papaya leaves[15].

5. In-Vitro Studies:

Caco-2 cells were treated with varying concentrations of the polyherbal extract and methotrexate, incubated for 24 hours, followed by MTT reagent addition. After crystal formation, DMSO was added to dissolve formazan, and absorbance was measured at 570 nm to calculate cell viability and inhibition[1,17].

Cell viability assessment using MTT assay:

The MTT assay for cell viability involves seeding cells into a 96-well plate, followed by treatment with the test compound for 24-72 hours. After treatment, 10 µL of 5 mg/mL MTT solution is added to each well and incubated for 3-4 hours at 37°C. Viable cells reduce MTT to purple formazan crystals, which are then solubilized by adding 100 µL of DMSO or isopropanol. The absorbance of the resulting solution is measured at 570 nm using a microplate reader. Cell viability is calculated by comparing the absorbance of treated wells to control wells using the formula:

$$\text{Viability (\%)} = \left(\frac{\text{Absorbance of treated}}{\text{Absorbance of control}} \right) \times 100.$$

6. RESULT AND DISCUSSION:

Table No.3. Qualitative Analysis of Polyherbal Powder Samples

Parameter	Hibiscus Petals	Neem Leaves	Papaya Leaves	Observation
Total Ash	Moderate	Highest	Moderate	Indicates high mineral content in Neem leaves
Acid-Insoluble Ash	Lowest	Moderate	Slightly higher	Minor soil residue in Papaya leaves
Water-Soluble Ash	Uniform	Uniform	Uniform	Consistent mineral solubility across all samples
Moisture Content	Low	Slightly higher	Moderate	Within acceptable limits, ensuring stability

6.3. MTT Assay Results of Polyherbal Extract and Standard on Caco-2 Cell Line:

6.1. Phytochemical Screening by Chemical Test:

The polyherbal decoction prepared from Hibiscus rosa-sinensis petals, Azadirachta indica leaves, and Carica papaya leaves showed positive results for major phytochemical constituents. Alkaloids, flavonoids, glycosides, carbohydrates, and steroids were all present, confirming the extract's richness in bioactive compounds responsible for antioxidant and anticancer properties.

Table No.2. Phytochemical Screening by Chemical Test

TEST	Observation
Alkaloids (Mayer's Test)	Present
Flavonoids (Shinoda Test)	Present
Glycosides (Keller Kiliani Test)	Present
Carbohydrates (Molisch's Test)	Present
Steroids (Liebermann- Burchard)	Present
Tannins (FeCl ₃ Test)	Present

6.2. Qualitative Analysis of Polyherbal Powder Samples:

Physicochemical analysis revealed that Neem leaves exhibited the highest total ash value, indicating high mineral content. The acid-insoluble ash was lowest in Hibiscus petals and slightly higher in Papaya leaves due to minor soil residue. The water-soluble ash values were uniform across all samples, suggesting consistent mineral solubility suitable for extraction. Moisture content was within acceptable limits, with Neem showing slightly higher values, confirming sample stability and purity.

The MTT assay results revealed a dose-dependent decrease in Caco-2 cell viability for both the



polyherbal extract and the standard drug methotrexate. The polyherbal extract exhibited progressive cytotoxic activity, reaching 18.83% inhibition at 200 µg/mL, indicating strong anticancer potential. Although slightly lower than methotrexate,

the extract maintained significant cell growth suppression with higher concentrations, confirming its promising antiproliferative effect due to synergistic phytochemical interactions.

Table No.4: MTT Assay Results of Polyherbal Extract and Standard on Caco-2 Cell Line.

Sr. No.	Sample Code	Conc. (µg/ml)	Optical Density			Mean	% Of Inhibition n (%)	% of Viability (%)
1	Standard Methotrexate	12.5	1.203	1.205	1.203	1.203	1.069%	98.93%
		25	1.196	1.195	1.196	1.196	1.644%	98.35%
		50	1.183	1.182	1.184	1.183	2.713%	97.28%
		100	1.176	1.174	1.175	1.175	3.371%	96.62%
		200	1.160	1.162	1.160	1.160	4.605%	95.39%
2	Polyherbal Extract	12.5	1.172	1.173	1.170	1.172	3.618%	96.38%
		25	1.152	1.150	1.151	1.151	5.345%	94.65%
		50	1.095	1.093	1.092	1.093	10.11%	89.89%
		100	1.043	1.044	1.042	1.043	14.22%	85.78%
		200	0.988	0.986	0.987	0.987	18.83%	81.17%

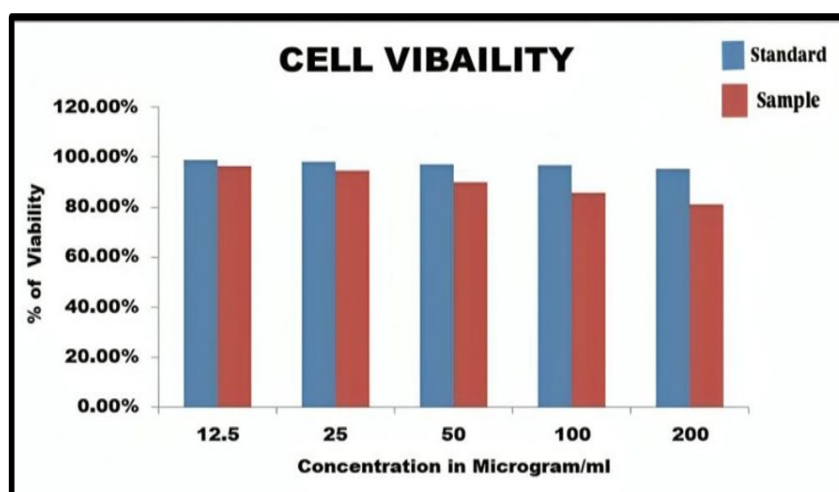


Figure No1.: The graphical representation of effect of Aqueous Polyherbal Extract and standard drug methotrexate by lipopolysaccharide induced inflammation on Caco-2 cell line by MTT assay.

The consequences of an aqueous polyherbal extract of performed by MTT assay by lipopolysaccharide induced inflammation on Caco-2 cell line with concentration 12.5, 25, 50, 100, 200 microgram/ml and methotrexate were a common medication. The effect of aqueous polyherbal extract also shows the high percentage of viability against lipopolysaccharide induced inflammation on Caco-2 cell line as comparable to standard drug methotrexate.

CONCLUSION:

The study demonstrated that the polyherbal decoction prepared from *Hibiscus rosa-sinensis* petals, *Azadirachta indica* leaves, and *Carica papaya* leaves

possesses promising anticancer potential against the Caco-2 colon cancer cell line. Physicochemical evaluations, including total ash, acid-insoluble ash, water-soluble ash, and moisture content, were within pharmacopoeial limits, confirming the purity, quality, and stability of the raw plant materials. The total ash value of 15 g samples incinerated at 550 °C indicated essential inorganic content with negligible contamination. Preliminary phytochemical tests confirmed the presence of compounds, verifying that all chemical tests were successfully passed and the extract contained active constituents contributing to its biological activity. In the MTT assay, the polyherbal extract produced a concentration-



dependent decrease in cell viability, with significant cytotoxic effects at 100–200 µg/ml compared with the standard. This indicates potent antiproliferative action likely due to synergistic phytochemical interactions. Overall, the results proved that the extract met all physicochemical and phytochemical standards and exhibited effective anticancer potential. The findings validate traditional herbal claims and support further in-vivo and mechanistic studies to develop safe, natural, and affordable anticancer formulations.

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