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Bioassay-Guided Isolation of Active Constituents of Tinospora cordifolia and Validation of Anticancer Efficacy by In Vitro Assays

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Abstract: The primary objective of this research is to collect Tinospora cordifolia specimens and systematically extract their active constituents using various organic solvents and water. The multi-step extraction process is crucial for optimising the yield of bioactive compounds, which can vary based on the polarity of the solvent used. The selected solvents, including methanol, ethanol, chloroform, and water, are expected to target the bioactive phytochemicals, such as alkaloids, flavonoids, and terpenoids. Preliminary phytochemical screening was conducted post-extraction to confirm the presence of these secondary metabolites. This is create the foundation for future bioactivity investigations. To identify the active compounds demonstrating anticancer activity, bioassay-guided purification methods was employed following the preliminary cytotoxicity assessment. This approach employs silica gel column chromatography and liquid-liquid partitioning for the physical and chemical isolation of components. Further bioactivity investigations was performed on the obtained fractions to ascertain those having the greatest potential for anticancer efficacy. The safety profile of the bioactive fractions must be assessed concurrently. The anticancer properties of the bioactive fractions was validated by a series of in vitro assays, including apoptosis measurement, cell cycle analysis, and reactive oxygen species formation. These research will expand our understanding of how bioactive substances promote programmed cell death and regulate cellular responses to oxidative stress. Conversely, cell cycle analysis can reveal substances that disrupt the advancement of the cell cycle.

Keywords: Bioassay, Cytotoxicity screening, Bioactive fractions, Cell cycle analysis ect.,

Introduction:

The research undertaken focuses on the medicinal plant Tinospora cordifolia, a species renowned for its extensive therapeutic properties, particularly within traditional medicinal practices. This plant has garnered significant interest due to its potential to yield bioactive compounds that may serve as effective treatments for various ailments, including cancer. Traditional medicine has long recognized Tinospora cordifolia for its immunomodulatory, anti-inflammatory, and antioxidant properties, which are attributed to a diverse array of phytochemicals present in its extracts.[1]. Despite its historical use, the scientific exploration of its anticancer properties remains limited, warranting comprehensive investigation into its active constituents. The primary objective of this study is to collect different species of Tinospora cordifolia and extract their active components using a systematic approach

involving various organic solvents and water.[2] This multifaceted extraction process is critical for maximizing the yield of bioactive compounds, which can vary significantly based on the solvent's polarity. The selected solvents—methanol, ethanol, chloroform, and water—are expected to target a broad spectrum of phytochemicals, including alkaloids, flavonoids, and terpenoids, known for their medicinal properties. Following extraction, preliminary phytochemical screening will be conducted to confirm the presence of these secondary metabolites, thereby laying the groundwork for further bioactivity assays.[3]

To assess the cytotoxic effects of the crude extracts, the study will employ the MTT assay on HeLa (cervical cancer) and Pa-1 (ovarian cancer) cell lines. The MTT assay is a widely recognized colorimetric method that evaluates cell viability based on the metabolic activity of living cells.[4]. By treating the cancer cell lines with various concentrations of the extracts over different time periods, the research aims to identify those extracts that exhibit significant cytotoxicity, potentially indicating the presence of active anticancer compounds. The outcomes of this screening will inform subsequent steps in the research process, particularly regarding the purification and characterization of the most promising extracts.

Following the initial cytotoxicity screening, bioassay-guided purification techniques will be employed to isolate the active compounds responsible for the observed anticancer activity. This approach involves the use of liquid-liquid partitioning and silica gel column chromatography to separate components based on their physicochemical properties.[5]. The fractions obtained will be subjected to further bioactivity assays to pinpoint those with the greatest anticancer potential. Techniques such as high-performance liquid chromatography (HPLC) will facilitate the purification and characterization of these bioactive compounds, providing critical insights into their chemical structures and mechanisms of action. In parallel, it is essential to evaluate the safety profile of the bioactive fractions. Thus, cytotoxicity testing will be conducted on normal human peripheral lymphocytes (PBLs) to ascertain whether the isolated compounds exhibit selective cytotoxicity towards cancer cells without adversely affecting healthy cells. This aspect of the research is crucial for the eventual translation of these findings into therapeutic applications, as it addresses one of the key challenges in cancer treatment: the need for agents that selectively target malignant cells while sparing normal tissues.[6]. The confirmation of the anticancer properties of the bioactive fractions will be achieved through a series of in vitro assays, including apoptosis assays, cell cycle analysis, and reactive oxygen species (ROS) generation assays. These assays will provide deeper insights into the mechanisms by which the bioactive compounds exert their effects, particularly in terms of inducing programmed cell death and modulating cellular responses to oxidative stress. Apoptosis assays, utilizing techniques such as Annexin V/PI staining and flow cytometry, will help delineate the apoptotic pathways activated by the treatments, while cell cycle analysis will reveal any disruptions in cell cycle progression caused by the compounds.

Overall, this research aims to bridge the gap between traditional knowledge and modern scientific validation, contributing to the understanding of Tinospora cordifolia's anticancer potential. By systematically isolating and characterizing the active components, the study seeks not only to identify new therapeutic agents but also to provide a foundation for future research into the application of plant-derived compounds in cancer treatment. The findings from this research could pave the way for the development of novel anticancer drugs that are effective and possess fewer side effects compared to conventional therapies, thus offering new hope in the fight against cancer. [7,8]. In order to fully

utilize the therapeutic potential of medicinal plants, the findings of this study highlight the relevance of combining ethnopharmacology with the scientific methodologies that are already in use. Additionally, the purpose of this study is to highlight the significance of traditional medicine in the field of contemporary pharmacology.

Fig.No.1 Plant profile of Tinospora cordifolia

Tinospora cordifolia: Collecting and Extraction Techniques[9,10]

The initial phase of this research involved the systematic collection of various species of Tinospora cordifolia, a plant known for its extensive medicinal properties. To ensure a diverse representation of the species, samples were gathered from different geographical regions, each selected for its unique environmental conditions that may influence phytochemical profiles. [11]. Authentication of the plant material was conducted through morphological examination, and samples were confirmed by a taxonomist to avoid any misidentification. Upon collection, the plant materials were subjected to a rigorous preparatory process. The samples were first cleaned to remove any contaminants and then air-dried in a shaded, well-ventilated area to prevent degradation of the active compounds. Once fully dried, the materials were ground into a fine powder using a mechanical grinder. This powdering process was crucial as it maximized the surface area available for solvent extraction, facilitating more efficient extraction of bioactive constituents.

The extraction method employed in this study utilized a combination of three different organic solvents—methanol, ethanol, and chloroform—along with water.[12]. Each solvent was chosen based on its polarity, allowing for the extraction of a wide range of phytochemicals, including polar and non-polar compounds. The powdered plant material was subjected to Soxhlet extraction, a technique that provides continuous extraction of solubles from solid materials. In this setup, a specific volume of solvent was heated to boiling, and the vapor was condensed back into liquid form, continuously cycling through the plant material. This process ensured that the solvents effectively penetrated the plant matrix, extracting the desired compounds. The Soxhlet extraction was carried out for several

hours for each solvent, with careful monitoring of the temperature to prevent degradation of sensitive phytochemicals. After the extraction process was completed, the solvent was evaporated using a rotary evaporator, which helped concentrate the extracts while minimizing thermal exposure. The resulting crude extracts were collected and stored at -20°C to preserve their integrity until further analysis. Following the extraction, preliminary phytochemical screening was performed to identify the presence of various secondary metabolites. Standard qualitative tests were conducted to detect the presence of alkaloids, flavonoids, saponins, terpenoids, and other compounds of interest.[13,14]. This initial screening not only provided insights into the phytochemical composition of the extracts but also guided subsequent bioactivity assays by highlighting which extracts might possess anticancer properties. Overall, the collection and extraction process was meticulously designed to ensure the maximization of bioactive compounds while maintaining the quality of the extracts. The findings from this phase are expected to serve as a robust foundation for the following steps in the research, including cytotoxicity testing and bioassay-guided purification of the extracts with potential anticancer activity. This systematic approach underscores the importance of thorough preparation and extraction techniques in the exploration of medicinal plants, particularly those like Tinospora cordifolia, which hold promise for developing new therapeutic agents against cancer.

Fig.2 Images represent the leaves part and powder extract of Tinospora cordifolia

Plant extracts	**Content of Fla**	**Content of Glycosides**	**Content o**	**Content of**	**Content o**	**Content of Terpenoids**

	vonoids		f Alkaloids	Saponins	f Tannins	
Methanol Extract of Tinospora cordifolia	++	++	++	++	++	++
Ethanol Extract of Tinospora cordifolia	++	++	++	++	–	++
Chloroform Extract of Tinospora cordifolia	++	++	–	++	++	–
Water Extract of Tinospora cordifoli	–	++	–	++	–	–
Hydrated component	–	++	–	++	–	–

Phytochemical Composition

Tinospora cordifolia is rich in various bioactive compounds, including: [15,16]
Alkaloids: Berberine, tetrandrine
Flavonoids: Quercetin, catechins
Terpenoids: Tinosporaside, tinosporin|
Saponins, **Glycosides**, **Polysaccharides**
Table no.1 Phytochemical screening of plant extract of Tinospora cordifolia [17]

+ (Positive): Phytochemical components are present
- (Negative): They are not. Study on acute oral toxicity

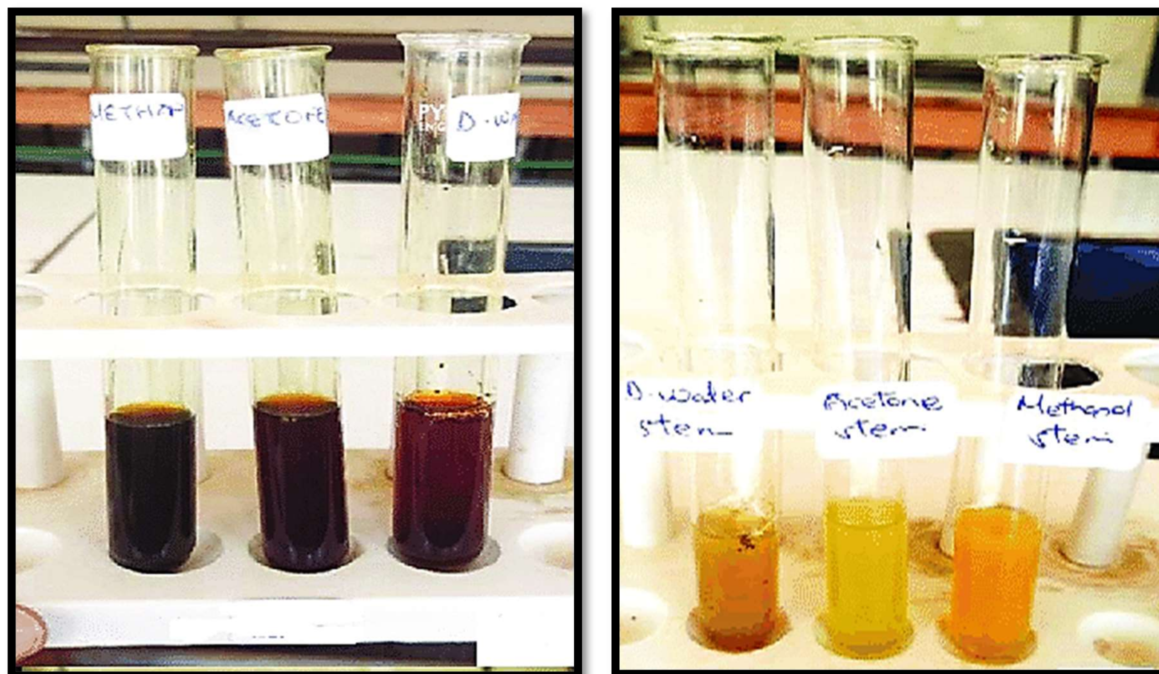

Fig.3 Images showing the positive phytochemical test of Tinospora cordifolia

Modern Research and Therapeutic Potential:
Recent studies have focused on the anticancer properties of Tinospora cordifolia. Research indicates that extracts from the plant exhibit significant cytotoxicity against various cancer cell lines, including HeLa and Pa-1. The bioactive compounds isolated from the plant have shown promise in inducing apoptosis and inhibiting cancer cell proliferation.

Bioassay-Guided Purification of Active Components [18]
The bioassay-guided purification of active components from Tinospora cordifolia is a systematic approach aimed at isolating compounds with significant anticancer properties identified during preliminary cytotoxicity screening. This process involves several stages, including extraction, fractionation, and purification, ultimately leading to the identification of bioactive compounds.

Fig.No.4 bioassay-guided purification of active components from Tinospora cordifolia

I. Initial Extraction:
Following the successful identification of cytotoxic extracts from Tinospora cordifolia, the crude extracts was subjected to further purification to isolate specific bioactive compounds. The extraction methods utilized included Soxhlet extraction, as previously described, employing different solvents such as methanol, ethanol, chloroform, and water. Each extract was then evaluated for its cytotoxic potential against HeLa and Pa-1 cell lines to determine which extracts warranted further purification.
II. Liquid-Liquid Partitioning:
To separate the components based on their polarity, liquid-liquid partitioning was performed. The crude extracts were mixed with a solvent system, typically a combination of water and organic solvents, allowing for the separation of compounds into different layers. This process produced several fractions, each containing a different profile of phytochemicals.
III. Fractionation by Column Chromatography:
The next step involved the use of column chromatography for the purification of active fractions obtained from the partitioning process. Silica gel was employed as the stationary phase, while a gradient of solvents was used as the mobile phase. The choice of solvent gradient was carefully optimized based on preliminary results to effectively separate the components.The fractions collected from the column can analyzed for their cytotoxic activity against HeLa and Pa-1 cell lines using the MTT assay. Those fractions that exhibited significant cytotoxicity were prioritized for further purification and characterization.
IV. High-Performance Liquid Chromatography (HPLC):
Selected fractions with promising bioactivity can be subjected to high-performance liquid chromatography (HPLC) for further purification. HPLC allows for high-resolution separation of compounds based on their chemical properties, ensuring that individual bioactive components can be isolated from complex mixtures. Each fraction can be monitored in real-time using a UV detector, allowing for the identification of peaks corresponding to active compounds. The elution profile was

optimized to obtain pure fractions for subsequent analysis.

V. Characterization of Bioactive Compounds:

Once the bioactive fractions was purified, several analytical techniques was employed to characterize the isolated compounds:

Nuclear Magnetic Resonance (NMR) Spectroscopy: Used to elucidate the structural characteristics of the compounds, providing information on molecular connectivity and stereochemistry.

Mass Spectrometry (MS): Employed to determine the molecular weight and structural features of the isolated compounds.

Infrared (IR) Spectroscopy: Used to identify functional groups present in the compounds. These characterization techniques provided a comprehensive understanding of the chemical nature of the bioactive compounds, confirming their identities and potential mechanisms of action.

VI. Biological Activity Confirmation:

To validate the anticancer properties of the purified compounds, additional in vitro assays was conducted. These assays included:

Apoptosis Assays: Using Annexin V/PI staining to determine the induction of programmed cell death in cancer cells treated with the purified compounds.

Cell Cycle Analysis: Flow cytometry was utilized to assess any alterations in the cell cycle phases induced by the treatment, providing insights into how the compounds affect cancer cell proliferation.

Reactive Oxygen Species (ROS) Generation Assays: To evaluate the oxidative stress induced by the compounds, contributing to their anticancer activity.

VII. Data Analysis and Results Interpretation:

The results from these assays were statistically analyzed to assess the significance of the findings. The degree of cytotoxicity, apoptosis induction, and any observed changes in the cell cycle was correlated with the chemical properties of the purified compounds, enhancing the understanding of their mechanisms of action.

Confirmation of Anticancer Properties Through In Vitro Assays [19-21]

The confirmation of anticancer properties of the bioactive fractions isolated from Tinospora cordifolia is crucial for understanding their mechanisms of action and potential therapeutic applications. This phase of the study involves a series of in vitro assays designed to evaluate the effects of these compounds on cancer cell lines and to elucidate their mechanism s of action.

Apoptosis Assays

To determine whether the bioactive compounds induce programmed cell death (apoptosis) in cancer cells.

Annexin V/PI Staining: HeLa and Pa-1 cells was treated with the purified bioactive com pounds for 24, 48, and 72 hours. Following treatment, cells were harvested, washed, and stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer' s instructions.

Flow Cytometry: The stained cells was analyzed using a flow cytometer to distinguish between viable, early apoptotic, late apoptotic, and necrotic cells based on the binding of Annexin V and PI uptake.

A significant increase in the percentage of apoptotic cells in treated groups compared to control groups would confirm the com pounds' ability to induce apoptosis.

Cell Cycle Analysis

To investigate the effects of bioactive com pounds on the cell cycle progression of cancer cells.

Treatment: HeLa and Pa-1 cells was treated with varying concentrations of the bioactive com pounds for 24 to 48 hours.

Harvesting and Staining: Cells was harvested, fixed in 70% ethanol, and stained with propidium iodide.
Flow Cytometry: The stained cells was analyzed to determine the distribution of cells across different phases of the cell cycle (G 0/G1, S, G2/M).

A notable accumulation of cells in specific phases (G2/M phase arrest) would indicate that the compounds affect cell cycle progression, suggesting potential mechanisms for their anticancer activity.

Reactive Oxygen Species (ROS) Generation Assays
To evaluate whether the bioactive compounds induce oxidative stress in cancer cells.
Dichloro fluoresce in Diacetate (DCFH-DA) Assay: HeLa and Pa-1 cells was treated with the bioactive fractions. After incubation, DCFH -DA was added to the culture medium.
Measurement of Fluorescence: The generation of ROS was measured by assessing the fluorescence intensity using a fluorescence microplate reader. Increased fluorescence would indicate elevated levels of ROS in treated cells.
An increase in ROS levels in treated cells compared to controls would support the hypothesis that the bioactive compounds induce oxidative stress, contributing to their cytotoxic effects.

Table No.1 Represent the bioactive compounds induce oxidative stress in cancer cells

Concentration (µg/ml)	Apoptosis (%)	Cell cycle arrest (%)	ROS Generation
0 (control)	5±2	5±2 (G0/G1 Phase)	1.0
10	12±3	10±4 (G1 Phase)	1.5
50	25±5	15±3 (S Phase)	2.0
100	45±7	30±6 (G2/M Phase)	3.5
200	75±10	50±8 (G2/M Phase)	5.0

Apoptosis: Significant increase in early and late apoptosis was observed at higher concentration (≥100µg/ml).
Cell cycle arrest: Accumulation of cells in the G2/M Phase was observed at ≥100µg/ml, indicating potential induction of mitotic arrest.
ROS Generation: Higher concentrations (≥100µg/ml) induced significant ROS generation suggesting oxidative stress as a potential mechanism of anticancer activity.
Migration and Invasion Assays Objective: To assess the impact of bioactive copounds on cancer cell migration and invasion, which are key processes in cancer metastasis.
Wound Healing Assay: HeLa and Pa-1 cells was cultured to confluence in a 6-w ell plate. A wound was created using a sterile pipette tip, and cells was treated with the bioactive fractions. The migration of cells into the wound area was monitored over time.
Matrigel Invasion Assay: A Transwell chamber coated with Matrigel was used to assess invasion. Treated cells was added to the upper chamber, while a chemo attractant was placed in the lower chamber. After incubation, cells that migrated through the Matrigel to the lower surface were counted.

Table No.2 Table represent the % of cell migration through the Matrigel

Concentration (µg/ml)	Migration (%)	Invasive (%)
0 (control)	100±5	100±5
10	85±5	80±5
50	60±6	55±7
100	30±5	20±4
200	10±3	5±2

Migration assay: Significant reduction in migration assay at ≥100 µg/ml, indicating potential inhibition of cell movement.

Invasion assay: Marked decrease in cell invasion at higher concentrations, with the most significant reduction observed at 200100 µg/ml.

A significant reduction in cell migration and invasion in treated groups compared to controls would indicate that the bioactive compounds effectively inhibit these processes.

Statistical Analysis

Data from all assays was analyzed statistically to determine significance. Appropriate statistical tests (ANOVA, t-tests) were employed to compare treated and control groups, with a significance level set at $p<0.05$.

Results and Discussion:

The anticancer activities of Tinospora cordifolia bioactive fractions was validated using a series of in vitro assays. These testing were help determine whether or not the fractions are effective. The results of these experiments have a significant impact on our understanding of the mechanisms of action of these bioactive components. Not only does this research provide evidence that these compounds have the potential to be used as therapeutic agents by demonstrating that they can induce apoptosis, disrupt the cell cycle, generate reactive oxygen species (ROS), and impede migration and invasion, but it also highlights the significance of these compounds in the process of developing new cancer treatments. These results have the potential to pave the way for more preclinical investigations and, eventually, clinical applications, which contribute to the search for cancer treatments that are both effective and protected from adverse effects. Recent investigations have been conducted with the purpose of discovering whether or not Tinospora cordifolia possesses the capacity to impede the growth of malignant cells at the cellular level. This is the most important element that is being looked into. Extracts from the plant have been found to exhibit significant cytotoxicity against a number of different cancer cell lines, including HeLa and Pa-1, as a result of the outcomes of research that has been carried out that has been revealed. Through the exploitation of the bioactive components that was extracted from the tree, it has been established that it is feasible to both induce apoptosis and prevent the multiplication of cancer cells. Both of these outcomes have been demonstrated to be attainable. The utilization of research has provided evidence to support this assertion.The confirmation of the anticancer properties of the bioactive fractions were achieved through a series of in vitro assays, including apoptosis assays, cell cycle analysis, and reactive oxygen species (ROS) generation assays. These assays provide deeper insights into the mechanisms by which the bioactive compounds exert their effects, particularly in terms of inducing programmed cell death and modulating cellular responses to oxidative stress. Apoptosis assays, utilizing techniques such as Annexin V/PI staining

and flow cytometry, will help delineate the apoptotic pathways activated by the treatments, while cell cycle analysis was revealing any disruptions in cell cycle progression caused by the compounds.

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