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A Phytochemical Screening for Cytotoxicity of the Crude Extract of Tinospora cordifolia on HeLa and PA-1 Cell Lines Utilizing the MTT Assay

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Abstract: 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. 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. 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. 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. This study highlights the importance of integrating ethnopharmacology with contemporary scientific methodologies to unlock the full therapeutic potential of medicinal plants, reaffirming the relevance of traditional medicine in modern pharmacology.

Keywords: MTT assay, HeLa, Pa-1, karkinos, Metastasis, Metastasis, carcinogens ect.,

Introduction:

Women give birth to all human life on the globe. Since the beginning of time, women have been seen as the more powerful gender. This is due to research indicating that women can also live longer than 80 years. In a sense, this is accurate. However, we also can't ignore the reality that women experience a variety of health issues throughout their lives [1]. While some are very particular, others are rather common. Cancer is the most important of these health problems. Any region of the body can be affected by a wide range of disorders collectively referred to as cancer. Uncontrolled proliferation of immature cells is cancer [2]. These cells proliferate and divide uncontrollably, invading distant organs directly or through the blood or lymphatic systems. Cancer is a major cause of death and can have serious health effects. Cancers of the breast, lungs, uterus, cervix, and stomach are most common among women. 2012's National Cancer Institute. In developing nations, cervical cancer is the leading cause of cancer-related mortality and the second most common malignancy among women. A group of illnesses known as cancer are defined by unchecked cell proliferation

[3]. Cancers have the ability to spread, disrupt the neurological, gastrointestinal, and circulatory systems, and release hormones that change how the body works. In general, tumours that remain stationary and show little growth are regarded as benign. Malignant, or more hazardous, tumours develop when two things take place: Through the use of the blood or lymphatic systems, a malignant cell is able to spread throughout the body, destroying good tissue in a process known as invasion [4]. The cell also has the ability to divide and develop, producing new blood vessels in angiogenesis, which is the mechanism by which it feeds itself. A tumour is said to have metastasized when it has successfully travelled to other areas of the body, grown, and invaded and destroyed other healthy tissues. Metastasis is the term for this process, which leads to a dangerous illness that is extremely challenging to cure. Cancer is the unchecked growth of cells due to a variety of unknown and little-known hereditary and environmental causes. Cell signalling networks in normal cells are frequently balanced, allowing them to precisely predict when a cell will proliferate or die [5].

Origin of Cancer:

The disease cancer is not new. Written accounts of it from approximately 1600 BC can be found on Egyptian papyrus. Hippocrates, the Greek physician, and father of medicine, is credited with coining the term "cancer." Hippocrates named cancer "karkinos" because he used the Greek terms carcinos and carcinoma to refer to tumours. Hippocrates believed a tumour to resemble a crab, so the Greek phrases were actually words to describe that creature. Hippocrates may have given the sickness its name, but he was by no means the first to identify it. The field of oncology, the scientific study of cancer, began with the execution of the first autopsy in 1761 by Italian anatomist Giovanni Morgagni [6].

History of Tumour:

A recent large-scale study that examined over 10,000 specimens of dinosaur vertebrae for tumours using fluoroscopy and further evaluated abnormalities using computerised tomography (CT) may provide the most convincing evidence of cancer in dinosaurs. Only cretaceous hadrosaurs, or duck-billed dinosaurs, who lived approximately 70 million years ago, had benign tumours such as hemangiomas, desmoplastic fibromas, and osteoblastomas out of the various dinosaur species examined; nonetheless, 0.2% of specimens showed malignant metastatic illness [7,8].

Epidemiology:

In addition to estimating the annual number of new cases and deaths caused by cancer, the American Cancer Society also compiles the most recent data on cancer incidence and outcomes based on the nation's population. With 350 daily fatalities, lung cancer is by far the leading cancer killer. Between 2014 and 2018, the incidence of prostate cancer stayed constant, while the incidence of female breast cancer continued to climb modestly at a rate of 0.5% per year [11]. This is in contrast to the 4% to 6% yearly increase in advanced disease that has been observed since 2011. The outcome was an increase from 3.9% to 8.2% in the proportion of prostate cancer patients detected at an advanced stage throughout the past decade. The frequency of advanced lung cancer kept falling quickly, whereas the rate of localized-stage lung cancer rose sharply by 4.5% every year. Because of this, the percentage of cases with localized-stage lung cancer were higher in 2018 (28% vs. 17%) and 3-year relative survival was higher (31% vs. 21%) [12–14].

Cancer development phases:

A malignant tumour develops through three distinct phases. In the first step, a DNA mutation is created by either incorrect or nonexistent DNA repair. [15] In the second stage, known as promotion, measures are taken to facilitate the unregulated proliferation of mutant cells. After a while, these cells just proliferate without any ability to do anything else [16]. This involves the metastasis of cancer cells to nearby tissues as well as their dissemination via the bloodstream and other transport mechanisms. Substances introduced to the body have the potential to either stimulate change or cause mutations. These substances are known as "complete carcinogens" since they can induce both types of cancer. Inducing cell division, some mutagens damage neighboring tissues. There is a chance that this could cause cancer [17].

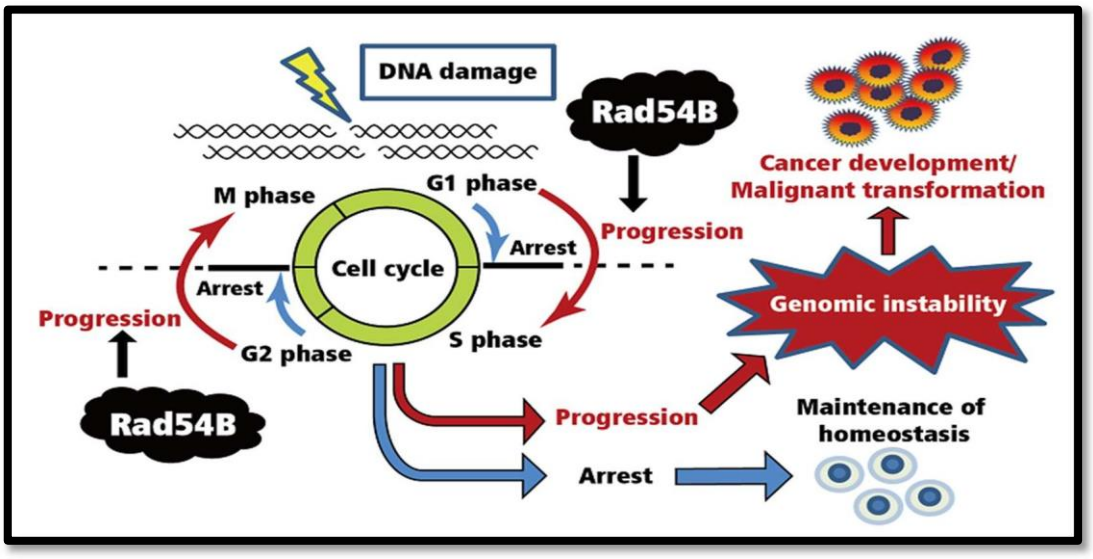


Fig No. 1 Flow diagram showing the mechanism of Cancer

The cell either gets rid of or stores the byproducts of the metabolic process when it absorbs chemical carcinogens. Gene expression and regulation involved in cell-cycle control, DNA repair, cell differentiation, and apoptosis can be directly or indirectly impacted by carcinogens or their metabolic byproducts within the cell. Some carcinogens have multiple genotoxic modes of action, including chromosomal breakage, fusion, deletion, mis-segregation, and non-disjunction [18]. The hallmark characteristics of cancer cells, such as hypermutability, genomic instability, loss of control over proliferation, and resistance to apoptosis, may eventually develop as a result of the combination of these genotoxic and nongenotoxic mechanisms, which may alter signal-transduction pathways [19].

Classification of Cancer:

Cancer is categorized into five major categories [20]: -

1. Cancers of the breast, colon, and lungs are examples of internal and external bodily parts covered by cancerous cells.
2. Cells found in bone, cartilage, fat, connective tissue, muscle, and other supporting tissues are what define sarcomas.
3. Cancers called lymphomas start in immune system tissues and lymph nodes.
4. Cancers called leukaemia start in the bone marrow and frequently spread throughout the blood.

5. Cancers known as adenomas can develop in the pituitary, adrenal, thyroid, and other glandular tissues.[21-22].

One cell is the source of cancer. It usually takes several stages for a normal cell to turn into a tumour, progressing from a precancerous lesion to malignant tumours. These alterations are the outcome of the interplay between three types of external agents and an individual's genetic makeup, such as: biological carcinogens, such as infections from specific viruses, bacteria, or parasites, and physical carcinogens, such as ultraviolet and ionising radiation. viruses include human papilloma virus (HPV) and cervical cancer, human immunodeficiency virus (HIV) and Kaposi sarcoma, and hepatitis B and liver cancer. Gastritis and Helicobacter pylori are examples of bacteria [23]. Cancer of the colon Limited evidence for ionising radiation and the solvents toluene and xylene. Hodgkin's illness Pesticides, woodworking, and solvents [24]. Excesses in the manufacturing of mustard gas and chemicals, rubber workers and nickel refining. Blood cancer Paints and pigments; organic and chlorinated solvents; reactive chemicals; ionising radiation; contradictory data regarding non-ionizing radiation; insecticides. biliary and liver cancer PCBs, vinyl chloride, and liver angiosarcoma; ionising radiation. There is some evidence for reactive compounds, arsenic, and chlorinated solvents [25]. There is some evidence linking dye products, insecticides, ionising radiation, and solvents. Nasopharyngeal and nasal cancer Ionising radiation; natural fibres, such as wood dust; chromium; nickel; metalworking fluids; benzene, formaldehyde, and reactive compounds may be present in trace amounts. connected to employment in the manufacturing of shoes. Lymphoma non-Hodgkin's insecticides and organic solvents. There is some evidence of dioxin, PCBs, and potentially dye products [26]. There is little proof of an overabundance of beauticians and hairdressers. stomach cancer Mineral oils, metalworking fluids, and acrylicamide. There is some proof of solvent exposure, cadmium, nickel, reactive compounds, and maybe formaldehyde. Little proof exists regarding pesticides. Testicular cancer connections to some herbicides, arsenic, and cadmium [27]. Colorectal cancer Mineral oils and fluids for metalworking. Some proof that solvents, such as xylene and toluene, exist. Skin cancer. UV and sun exposure; mineral oils and metalworking fluids; skin cancers other than melanoma caused by ionising radiation, coal tar, arsenic, creosote, and PAHs. Sharp tissue tumours Pesticides; vinyl chloride monomer (hepatic angiosarcoma) [28]. Some proof that solvents and pesticides work. Workers in the rubber, coal, iron, lead, zinc, and gold mining industries have higher risks than average. Cervical cancer Evidence of substances that damage the endocrine system, such as polyhalogenated hydrocarbons, PCBs, and phthalates [29].

Ovarian cancer:

In the first step of two-stage techniques, more sensitivity is needed for better detection, while very high specificity is maintained. Although they do not offer lead time, HE4 and CA72-4 antigens can detect around 16% of early-stage ovarian tumors that CA125 misses. Autoantibodies against TP53 can be found in 20% of patients with high-grade serous ovarian tumors; these autoantibodies typically rise 8 months before CA125 and 22 months before diagnosis in patients with normal CA125, respectively. Almost all of these malignancies had TP53 mutations. 39% of patients with early-stage ovarian cancer had HE4 antigen-autoantibody complexes in their serum, 62% had high CA125, and 80% had both [30].

Vaginal Cancer:

The cervix is the opening of the uterus, and the vagina is the passageway that extends from that opening to the outside world. The vagina, often known as the birth canal, is the main exit point for the infant during birth.

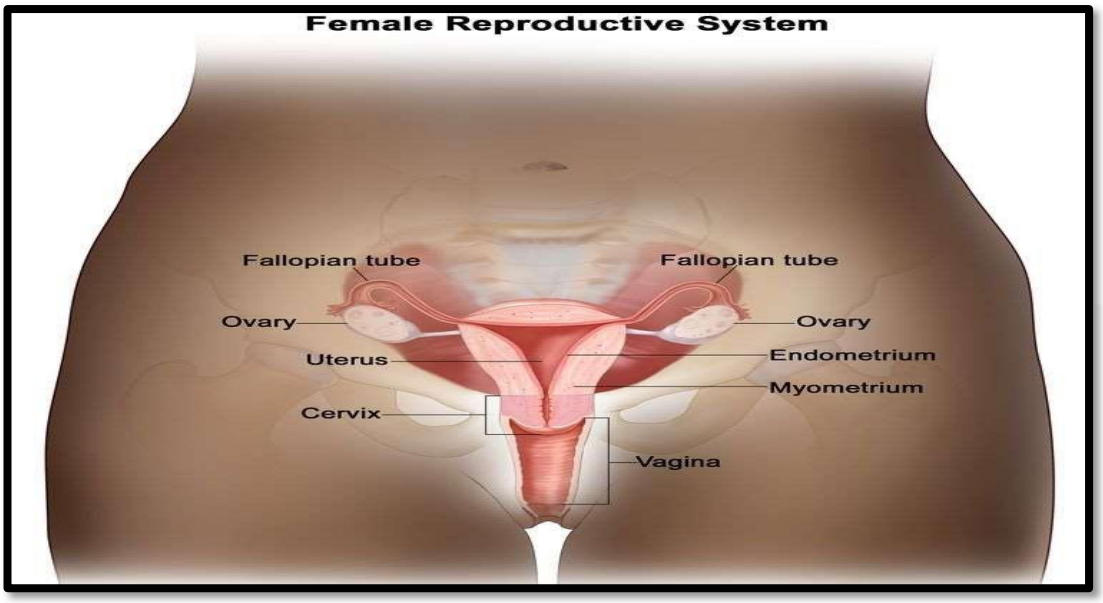


Fig.No.2 Diagram represent the Female reproductive system

Anatomy of the female reproductive system. The organs in the female reproductive system include the uterus, ovaries, fallopian tubes, cervix, and vagina. The uterus has a muscular outer layer called the myometrium and an inner lining called the endometrium [31].

There are two main types of vaginal cancer:[32]

For women, the two most common forms of vaginal cancer are: [32]

Cancer that develops in the flat, thin cells that line the inside of the vagina is called squamous cell carcinoma. Although it typically remains localized to the vagina, squamous cell vaginal cancer can slowly metastasize to other organs such as the liver, lungs, or bones. This particular form of vaginal cancer is quite prevalent.

The malignancy known as adenocarcinoma originates in the cells of the glands. The glandular cells found in the vaginal lining are responsible for producing and secreting mucus. Adenocarcinoma is more likely to metastasize (spread to other organs) to the lymph nodes and lungs than squamous cell cancer. Adenocarcinoma with clear cell characteristics is an extremely rare tumor that has been linked to diethylstilbestrol (DES) exposure during pregnancy. There is no correlation between DES use and the increased risk of adenocarcinomas in postmenopausal women.

Material and Methods: [33]

Chemical requirements:

Tinospora tuberculate, Tinospora sagittate, Tinospora sinensis, HeLa and Pa-1 Cell Lines, methanol, ethanol, and chloroform, distilled water, DMSO, MTT solution, Soxhlet apparatus and rotary evaporator.

Collection and identification of plant species:

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 profile. Authentication of the plant material was conducted through morphological examination, and samples were confirmed by taxonomist Dr Arti Garg at Botanical Survey of India, Prayagraj with specimen ref. no. 2022-23/01762.

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 a rea available for solvent extraction, facilitating more efficient extraction of bioactive constituents.

Extraction of the plant:

The extraction method employed in this study utilized a com bination of three different organic solvents- methanol, ethanol, and chloroform- a long with water. Each solvent was chosen based on its polarity, allowing for the extraction of a wide range of phytochemicals, including polar and non-polar com pounds. The powdered plant material was subjected to Soxhlet extraction, a technique that provides continuous extraction of soluble 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 com pounds. 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 [34].

Phytochemical preliminary screening:

Following the extraction, preliminary phytochemical screening was performed ed to identify the presence of various secondary metabolites. Standard qualitative tests were conducted to detect the presence of alkaloids, flavonoids, saponins, terpenoids, and other com pounds of interest. 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 [35,36].

Overall, the collection and extraction process was meticulously designed to ensure the maximization of bioactive com pounds 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.

In-Vivo Anticancer Activity:

Screening for Cytotoxicity on HeLa and Pa-1 Cell Lines:

The assessment of cytotoxicity is a critical step in evaluating the potential anti-cancer properties of bioactive com pounds extracted from *Tinospora cordifolia*. This study specifically focuses on screening the cytotoxic effects of the crude extracts on HeLa (cervical cancer) and Pa-1 (ovaria n cancer) cell lines using the M TT

assay, a widely utilized method for assessing cell viability [37].

Preparation of Cell Lines:

HeLa and Pa-1 cell lines were cultured under controlled laboratory conditions. HeLa cells, derived from cervical cancer tissue, and Pa-1 cells, obtained from ovarian cancer, were maintained in appropriate growth media supplemented with fetal bovine serum (FBS), antibiotics, and other essential nutrients to support optimal cell growth. The cells w ere regularly passaged to maintain their viability and were kept in an incubator at 37° C with a humidified atmosphere of 5% CO_2.

Extract Treatment:

Following the establishment of optimal cell densities, the cultured cells were treated with varying concentrations of the crude extracts obtained from *Tinospora cordifolia*. The extracts were prepared in a sterile manner, ensuring that any contamination was minimized. The treatment groups included several dilutions of the extracts, typically ranging from 1 µg/m L to 100 µg/m L, to assess dose-dependent effects on cell viability.

Each treatment group was accompanied by a control group consisting of untreated cells, which served a s a baseline for com parison. The treatments were performed in triplicate to ensure the reliability of the results.

Fig.No.3 Soxhlet assembly and crude extract of Tinospora cordifolia

MTT Assay Procedure:

The MTT assay was conducted as follows [38]:

1. Incubation: After treatment with the extracts, the cells w ere incubated for 24, 48, and 72 hours, allowing sufficient time for the extracts to exert their effects on the cells.
2. MTT Addition: Following the incubation period, MTT solution was added to each w ell. The MTT reagent is a yellow dye that is reduced by metabolically active cells to form purple form azan crystals.
3. Incubation with MTT: The plates w ere further incubate d for 2 to 4 hours to allow adequate time for the cells to metabolize the MTT.
4. Solubilization: After the incubation, the culture medium was rem oved, and DMSO was added to dissolve the form azan crystals. This step was crucial to convert the crystals into a soluble form for quantitative analysis.

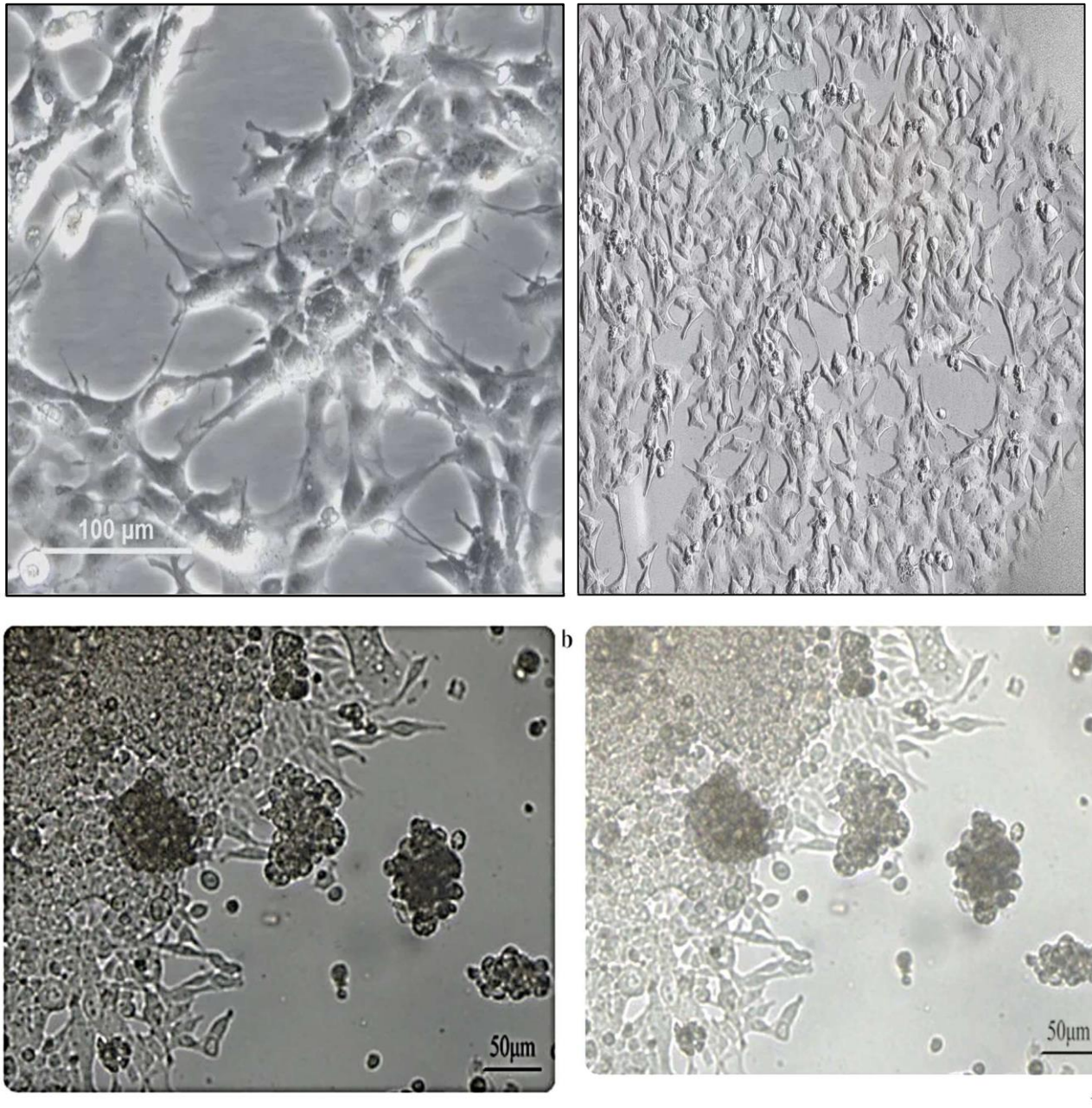
5. Measurement: The optical density (OD) of each w ell was measured at 570 nm using a microplate reader. The OD values correlate with the number of viable cells in each treatment group.
6. Data Analysis: The percentage of cell viability was calculated by com paring the OD values of treated cells to the control group using the formula

Data Analysis and Results Interpretation:

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

Fig.No.4 Showing microscopic image of cancer cell line obtained from ascitic fluid.

Treatment with Isolated Compounds:



Following the establishment of optimal cell densities, the isolated bioactive com pounds from *Tinospora cordifolia* were diluted to various concentrations for treatment. The concentrations were chosen based on previous cytotoxicity results against cancer cell lines, typically ranging from 1 µg/m L to 100 µg/mL. Each treatment group included multiple dilutions to evaluate the dose-response relationship. Control groups consisting of untreated lymphocytes were maintained for comparison.

Table No.1 bioactive compounds from *Tinospora cordifolia* were diluted to various concentration for treatment

Conc. (µg/ml)	Fraction 1 Viability (%)	Fraction 2 Viability (%)	Fraction 3 Viability (%)
0 (control)	100	100	100
10	95	90	92
50	80	75	78
100	50	60	55
200	20	30	25

IC_{50} Values:
Fraction 1: 150 µg/ml [Chloroform]
Fraction 2: 180 µg/ml [Ethyl acetate]
Fraction 3: 200 µg/ml [Methanol fraction]

MTT Assay Procedure:
To assess the cytotoxicity of the isolated com pounds on norm a l h um an PBLs, the MTT assay was utilized, as it provides a reliable measurement of cell viability. Th e procedure involved the following steps:
Incubation: Lymphocytes were incubated with the various concentrations of the bioactive com pounds for 24, 48, and 72 hours, allowing time for potential effects on cell viability.
MTT Addition: After incubation, MTT solution was added to each w ell. Viable cells reduced the MTT to purple form azan crystals.
Incubation with MTT: The plates w ere incubated for an additional 2 to 4 hours to facilitate the reduction of MTT by metabolically active cells
Solubilization: DMSO was added to dissolve the form azan crystals, converting them into a soluble form for measurement.
Measurement: The optical density (OD) was read a t 570 nm using a microplate reader to quantify cell viability.
Data Analysis: Cell viability percentages were calculated

Results Interpretation:
The results from the MTT assay provided crucial insights into the effects of the bioactive com pounds on norm al hum an PBLs. A high percentage of cell viability in treated groups compared to control indicated low cytotoxicity, while a significant reduction in viability suggested potential toxicity. The outcomes were analyzed for dose-dependent effects to ascertain whether higher concentrations of the com pounds correlate w ith increased cytotoxicity in norm al cells. Com parisons w ere also made with the results obtained from HeLa and Pa-1 cell lines to evaluate selectivity.

Statistical Analysis:

Statistical analysis was performed using appropriate software to determine the significance of the results. ANOVA and post-hoc tests were utilized to compare means across different treatment groups, with a significance level set at $p < 0.05$.

Table No.2 Determination of significance the results of different treatment group

Concentration (µg/ml)	HeLa viability (%)	PA-1 Viability (%)
0 (control)	100	100
10	95	93
50	75	70
100	50	45
200	20	15

IC_{50} Values

HeLa: 120 µg/ml PA-1: 150 µg/ml

Conclusion:

In general, these tumors are considered to be benign because they do not spread and do not exhibit any symptoms of development. This is the reason why they are considered to be benign. Both of these conditions, when present, are responsible for the formation of more dangerous tumors, which are typically referred to as malignant tumors: In the process that is known as invasion, a cancerous cell takes advantage of the lymphatic or circulatory systems in order to travel throughout the body and cause damage to healthy tissue. This is accomplished by using the lymphatic or circulatory systems. In addition, the cancerous cell goes through a process that is known as angiogenesis. This process involves the cell multiplying and developing in order to build new blood vessels that the cancerous cell can use to its advantage in order to feed itself. The term "metastasis" is used to describe the process by which a tumor is able to successfully spread to other parts of the body and has the potential to develop. As the tumor continues to expand, it will eventually invade and destroy other healthy tissues, which will lead to the emergence of this condition. Metastasis is the term that is used to describe the process itself, and this process ultimately results in a serious illness that is exceedingly difficult to treat. Some of the factors that contribute to the development of cancer are inherited, while others are environmental in nature. Cancer is characterized by the uncontrolled multiplication of cells, and it is caused by a number of different factors. One of the defining characteristics of cancer is an inability to exert control over the proliferation of cells. This is the second most significant cause of death that is brought on by a sickness, according to reports that have been gathered from all around the world. Several different sources have been consulted in order to compile this information. There are a number of characteristics that distinguish cancer cells, including uncontrolled cell proliferation, invasiveness, and metastasis. All of these characteristics are the result of cancer cells evading cell signaling pathways on various levels during their development. These traits are what distinguish cancer cells from other body cells. Because the cell signaling pathways that are present in normal cells are in a state of equilibrium, it is possible to exert precise control over the death or development of cells. This is because normal cells are also able to develop normally.

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