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A Comprehensive Review of *In vitro* Testing and Emerging Strategies Employed in Anticancer Drug Discovery Therapy

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Abstract:- Cancer is a broad category of diseases characterized through the unchecked proliferation and dissemination of atypical cells. It is among the primary causes of morbidity and mortality globally. The multistage genesis of cancer, which begins with genetic abnormalities that cause normal cells to become malignant, is what gives cancer its complexity. There are several steps involved in the genesis of cancer, including six key cancer distinguishing features known to influence malignant change has been identified. Anticancer drug development is a laborious process that includes numerous *in vitro*, *in vivo*, and clinical trials. In vitro assays provide a foundation for cancer medication development techniques. Numerous in vitro procedures and tests have been developed to analyze every defining aspect of cancer; the choice of a specific *in vitro* technique or assay is largely based on the research question(s) under investigation. Currently, oncology researchers are attempting to create cancer nanomedicines that are both safe and effective. While nanoparticles have opened up new therapeutic and diagnostic avenues, stem cell treatment has demonstrated potential usefulness in renewing and repairing defective or damaged tissues by tackling primary and metastatic cancer sites. With minimal harm to healthy cells, targeted therapy has the potential to stop the development and propagation of specific cancer cells. In place of open surgery, ablation therapy has become a popular minimally invasive method for destroying or freezing tumors. Naturally occurring antioxidants have demonstrated the ability to find free radicals and counteract their harmful effects, potentially treating or preventing cancer. A number of novel technologies have previously received authorization, and some are presently the subject of clinical trials. This review article's objective is to provide an extensive overview of the state of our knowledge on cancer, covering its causes, kinds, diagnosis, therapy, *in vitro* assays to screen cancer and most recent scientific developments.

Keywords:- *Cancer, In Vitro Assays, Modern Techniques, Herbal Therapy.*

I. INTRODUCTION

Cancer is caused by a series of abrupt gene changes that change cell signaling. It is clear that chemical compounds influence gene variations and the shape of cancer cells. Additionally, smoking has several chemical components that cause cancer, including lung cancer [1]. Fascinatingly, chemicals set up in the terrain that have the eventuality to beget cancer affect cells' cytoplasm and nexus either directly or laterally, performing in inheritable abnormalities and gene mutations [2-5]. Roughly 7 of all malice are caused by contagions, bacteria, and radiation exposure [6]. Cancer generally causes cellular connections to break down and essential genes to stop working. This dislocation causes aberrant proliferation and has an impact on the cell cycle [7, 8]. Proto-oncogenes normally stimulate the division and proliferation of cells, nonetheless, upon inheritable mutation, they transfigure into oncogenes, which pose the topmost trouble to cellular viability [9]. Both internal (inheritable mutations, vulnerable system diseases, and hormones) and external (salutary, radiation, tobacco use, and contagious organisms) variables can beget cancer

II. THE MITOTIC PHASE

Proteins that regulate the cell cycle's progression through its several phases gradually activate and deactivate, causing mammalian cell division. The cell cycle is comprised of G0 (gap 0), G1, S (synthesis), G2, and M (mitosis) phases. Mitosis contains five stages: prophase, metaphase, anaphase and telophase. To make sure that only healthy cells proliferate, checkpoints that trigger cell-cycle arrest have evolved in response to the discovery of defects that may have arisen during the replication of DNA or other phases leading to mitosis. These defects can be corrected by cell-cycle arrest, ensuring that every daughter cell inherits a healthy genome. Another critical function of cell-cycle checkpoints is their capacity to effectively trigger processes (such as death, mitotic catastrophe, and aging) to prevent the propagation of severely damaged or high-risk cells. Numerous checkpoints have set protocols in place. The G1/S checkpoint stops cells with DNA that has been damaged from entering S phase and

either induces senescence or cell death till the damage and associated risks are removed. The intra-S checkpoints slow down replication of DNA during S phase or delays replication origin firing in order to minimize replication mistakes. The G2/M checkpoint delays the premature entry of cells into mitosis, which lowers the amount of chromosome mis segregation. The spindle assembly checkpoint (SAC), also known as the mitotic checkpoint, is the main cell-cycle governing mechanism in mitosis. It is in charge of maintaining the integrity of chromosome segregation, which results in the generation of daughter cells that are genetically identical. Abnormal mitotic daughter cells are prevented from moving on to the next interphase by the post-mitotic checkpoint. For the cell cycle to progress with less genomic instability, all of these checkpoints are necessary.

➢ *Proliferation*

Cell proliferation is the pace at which a cancerous cell splits into two and copies its DNA. In cancer, rapid cell division suggests a more aggressive or faster-growing type of the disease. Proliferation is particularly described by cell count as a function of time; a metric utilized to quantify a growth of cells. As cells divide, they go through a sequence of events called as the mitotic cycle. Other names for the mitotic cycle include the proliferative cycle, generation cycle, and cell cycle. The trait of cancer cells that accelerates their growth and progression is called cell proliferation. Cell cycle proteins are expressed differently during proliferation. It can also be induced by signal-transducing pathways constitutively expressing themselves. A hypoxic environment and a fibrogenic reaction promote the expansion of cancerous cells. Cell proliferation is the process by which a tumor starts off small and spreads to other areas. This process is aided by the production of several hormones, which intensifies cancer. The epithelial to mesenchymal transition is the cause of this further rise in angiogenesis.

➢ *Cancer Types*

Cancer comes in more than a hundred varieties. Different cancer types are sometimes called after the organs or apkins in which the excrescences originate. As an example, lung cancer originates in the lung, but tumors in the brain originates in the brain.

➢ *Melanoma*

The most frequent type of cancer is melanoma. Their production is credited to epithelial cells, which envelop the body's external and internal shells. Adenocarcinomas are the mature form of malignancies that affect the prostate, colon, and bones.

➢ *Sarcoma*

Sarcomas are cancers that can arise in the stringy towel (similar as ligaments and tendons) and soft apkins of the bone, including the muscles, fat, blood, and lymph highways.

➢ *Leukemia*

Leukemias are cancers that show up in the bone marrow's blood-forming tissue. Solid excrescences are not produced by these tumors. Instead, when a sizable number of aberrant white blood cells—leukemia cells and leukemic blast cells—absorb in the bone structure and rotate, healthy blood cells are displaced. Cancerous lymph knots Cancer that starts in lymphocytes is known as lymphoblasts (T lymphocytes or B cells). Abnormal lymphocytes are found in lymph highways, lymph bumps, and other bodily organs in cancer.

➢ *The Study of Epidemiology*

The most recent estimates of the global cancer burden have been released by the World Health Organization's (WHO) cancer exploration association, the International Agency for Research on Cancer (IARC). In 2022, there are expected to be twenty million new instances of cancer and 9.7 million die from the disease. Five times after receiving a cancer diagnosis, 53.5 million patients were given a prognosis. Cancer affects one in five people at some point in their life; one in nine men and one in twelve women die from the disease. The three most common cancers in women are lung, colorectal, and bone; by 2024, these three cancer types will account for 51 of all new cancer diagnoses in this demographic. In 2024, 14,910 children and adolescents between the ages of 0 and 19 are expected to receive a cancer diagnosis; 1,590 of them will die from the disease.

III. REASONS FOR CANCER CAUSE

➢ *Cigarette*

in LMCs, tobacco use accounted for over one-fifth of all cancer deaths in 2002, making it the single biggest factor to cancer mortality. In addition, it is the main factor contributing to death when all major noncommunicable diseases are combined The middle-income countries are in stages 2 or 3 (tobacco products use peaked and is now rising sharply), whereas the low-income countries are largely in stages 1 or 2 (tobacco use is still low but is rising, especially among men; tobacco-related deaths are low and are only now starting to climb).

The task of tobacco control is to change the course of events in those nations by deterring youth from starting to smoke and encouraging adults to stop (which has immediate benefits). This is the long-term solution.

➢ *Infections*

The three most prevalent cancer-causing infections in developing countries, in terms of associated risks for cancer incidence, are hepatitis B and hepatitis C viruses, human papillomavirus (HPV), and Helicobacter pylori. In terms of importance, tobacco comes in second. Getting vaccinated is a simple way to prevent HBV. The implementation of safe injection practices and blood bank screening is crucial in halting the transmission of hepatitis C; nevertheless, both interventions pose greater challenges.

➢ *Diet, Obesity and Overweight, and Inactivity*

Eating habits, body weight, and levels of physical activity seem to interact in a complicated way that either raises or lowers the probability of cancer. Except for legumes (peas and beans), which have a greater amount of carbohydrates than other vegetables, epidemiologic studies have consistently demonstrated a correlation between a high

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consumption of fresh fruits and vegetables and a decreased risk of cancer.

➢ *Alcohol Consumption*

Alcohol abuse has been associated with malignancies of the breast, liver, esophagus, larynx, and mouth. An estimated 5% of deaths from LMC cancer are thought to be due to this risk, which varies based on the cancer site and rises for every cancer site with increased intake.

➢ *Exposures at Work*

Factory floors, farms owned by families, city streets, and other workplaces can all be hazardous, formal or informal. 2 million deaths worldwide are attributed to occupational hazards, impacting 2.7 billion people, according to estimations from the International Labor Organization (ILO). An additional 25 chemicals have been classified as probably carcinogenic by the IARC, while around 25 substances or combinations of chemicals, most of which are job-related, have been shown to cause cancer in humans.

➢ *Signs and Symptoms*

These are a many of the most typical suggestions and symptoms that could be brought on by cancer.

- Inordinate fatigue or weariness that does not go down with rest.
- Unknown cause for weight gain or loss of at least ten pounds
- Issues related to eating, similar as not being empty, difficulty swallowing, abdominal pain, nausea, and puking
- Anywhere in the body swelling or lumps;
- Guts or other corridor of the body thickening or lumping;
- New or unexplained pain that does not go down or grows worse
- Skin abnormalities including a sore that will not go down, a lump that bleeds or becomes scaled, a new operative or a operative that changes, or yellowing of the skin or eyes (hostility).
- Unusual bleeding or bruising that doesn't go down;
- Constipation or diarrhea that doesn't go down;
- Changes in the appearance of your droppings;
- Bladder changes, similar as pain when passing urine, blood in the urine, or the need to pass urine more or less constantly;
- Fever or night sweats
- Headaches;
- Issues with vision or hail;
- Variations to the mouth, similar as ulcers, bleeding, pain, or impassiveness

IV. IN VITRO ASSAYS FOR SCREENING ANTICANCER DRUGS

➢ *Assay for DNA Fragmentation*

The DNA fragmentation assay is a crucial method in the investigation of cell death, or programmed killing of cells, and the evaluation of the effectiveness of anticancer treatments.

As a telltale sign of apoptosis, fragmented DNA is measured by this technique. A thorough description of the DNA fragmentation assay, its use in cancer research, and a list of references are provided below. [10]

➢ *Principle*

During apoptosis, DNA fragmentation occurs when the molecule splits into fragments that are either internucleosomal or oligonucleosomal [11]. There are other ways to find this fragmentation, such as:

- Agarose Gel Electrophoresis: On an agarose gel, fragmented DNA presents as a "ladder" pattern.
- TUNEL Assay (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling): This technique uses fluorescent or chromogenic tags to mark the ends of broken DNA.
- Comet Assay: This method identifies single-cell DNA breaks by observing how fragmented DNA leaves the cell during electrophoresis and forms a "comet tail."
- Cytoplasmic histone-associated DNA fragments are determined with the use of the ELISA (enzyme-linked immunosorbent assay). [12]

➢ *Procedure:*

- Cell Culture: Possible anticancer drugs are applied to cells.
- Induction of Apoptosis: Culture cells to facilitate the death process.
- Harvesting Cells: Use centrifugation to gather cells.
- Lysis: To liberate DNA, lyse the cells.
- Fragmentation Detection:
- Put the isolated DNA on an agarose gel for gel electrophoresis.
- To add labeled nucleotides for TUNEL, fix cells and employ terminal transferase.
- Place cells in agarose on a slide for the comet assay, then electrophorese.
- Use certain antibodies to find fragmented DNA for ELISA.

➢ *LDH Assay*

Lactate dehydrogenase (LDH) assays are widely used in cancer research to evaluate cell viability, cytotoxicity, and drug response. The LDH enzyme is found in almost all body cells. Its release into the extracellular area indicates lysis or damage to the cell membrane, which often indicates cell death. This article gives a general review of the LDH assay and how it is used in *in vitro* cancer studies [13].

- *Principle*

Lysed or damaged cells release LDH into the culture media, which is the underlying operating principle of the LDH test. Together with converting lactate to pyruvate, the enzyme catalyzes the reduction of NAD+ to NADH.It is possible to measure the amount of NADH generated, which is directly correlated to the LDH activity colorimetrically or fluorometrically [14].

- *Procedure*

Cell Culture: While cells are being cultivated in appropriate circumstances, potential anticancer medications are added to them. Therapy: For a specific period of time, the cells receive the therapy [15]. Supernatant Collection: After treatment, the culture medium—which contains the released LDH—is removed. Creating the Reaction Mix: In a reaction mix, combine lactate, NAD+, and an indicator dye (such INT or WST) that changes color when reduced by NADH. [17]. Incubation: Allow the mixture to remain for a while after adding the reaction mix to the collected supernatant. Measuring: Determine the absorbance or fluorescence of the reaction result using a plate reader [16].

- *SRB Test*

A tried-and-true technique for determining cell density and proliferation in cancer research is the Sulforhodamine B (SRB) assay. It is especially helpful for determining how different therapies affect the cytotoxicity and proliferation of cancer cells. An extensive synopsis of the SRB assay's use in in vitro cancer research is provided below [18].

- *Principle:*

The Sulforhodamine B protein dye's ability to bind to the basic amino acid residues of biological proteins is what allows the SRB assay to function in slightly acidic settings [19]. The quantity of dye attached to the cells, which has a direct relationship with the mass of the cells, is used to determine the density and vitality of the cells.

V. METHOD

- *Cell Culture:*

- A 96-well plate is seeded with cells, which are then allowed to adhere and proliferate.
- Therapy: For a predetermined amount of time, cells are given possible anticancer medications [20].
- Fixation: Cells are fixed by precipitating cellular proteins in the wells with trichloroacetic acid (TCA).
- Staining: SRB dye, which binds to the proteins in the cells, is used to stain the fixed cells. [21]
- Washing: Using acetic acid, unbound dye is eliminated. [22]
- Solubilization: Tris buffer is used to solubilize the dye that is bound to proteins.
- Measuring: A plate reader is used to measure the solubilized dye's absorbance at a wavelength of 564 nm.

- *Alamar Blue Test*

One popular technique for determining cell viability and cytotoxicity in in vitro cancer research is the Alamar Blue assay [23]. The method is predicated on the living cells' capacity to convert the non-fluorescent resazurin dye to the fluorescent resorufin, which serves as a sign of metabolism and, thus, of the vitality of cells [24]. Below is a summary of how the Alamar Blue assay is used in in vitro carcinoma research:

- *Principle:*

The blue, non-fluorescent redox indicator dye resazurin provides the basis for the Alamar Blue assay. Resazurin is broken down by viable cells into the pink, fluorescent substance resorufin. The decrease happens as a result of metabolic activity, which mostly uses enzymes found in the mitochondria. The quantity of viable cells determines how much resorufin is produced [25].

- *Procedure:*

Cell Culture: A 96-well plate is seeded with cells, which are then allowed to adhere and proliferate [26]. Therapy: For a predetermined amount of time, cells are given possible anticancer medications. Alamar Blue Addition: Resazurin, the Alamar Blue reagent, is applied to every well. Incubation: Resazurin is reduced to resorufin by living cells on the plate during incubation. Measuring: A plate reader is used to measure the decreased dye's absorbance (570 nm) or fluorescence (excitation/emission: 560/590 nm). [27]

- *Assay for Potato Disc Tumors*

The potato disc tumor assay is a special technique for assessing a substance's ability to cause tumors. It is especially useful for determining if chemical compounds, plant extracts, or microbes have anti-tumor qualities. It uses potato discs as a host tissue for Agrobacterium tumefaciens-induced crown gall tumors. A summary of the potato disc tumor assay's use in cancer research is provided here [28].

- *Principle:*

The principle of the potato disc tumor assay is the inoculation of potato discs with Agrobacterium tumefaciens, a bacterium that causes uncontrollable cell proliferation and tumor growth in plant cells by transferring a portion of its DNA to the cells. It is thought that substances that block this mechanism may have anti-tumor properties [29].

VI. PROCEDURE

Preparation of Potato Discs: After sterilizing fresh, disease-free potatoes, discs (approximately 8 mm in diameter and 2 mm thick) are cut with a knife and cork borer [30]. After briefly submerging the discs in a bleach solution, they are further disinfected by being rinsed with sterile distilled water. *Agrobacterium tumefaciens* inoculation: After preparing an *Agrobacterium tumefaciens* suspension, potato discs are submerged in the bacterial suspension for a short period of time. To get rid of extra germs, the infected discs are wiped on sterile filter paper. Test Compound Treatment: Inoculated potato discs are put on agar plates with different doses of the test chemicals. Plates devoid of the test substances are

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covered with control discs [31]. Incubation: Depending on the experiment, the plates are sealed and kept at 25°C in a growth chamber for two to four weeks. Evaluation: The potato discs are checked for tumor formation following the incubation period. Tumour size and quantity are tallied and documented for every disc [32]. The test chemicals' anti-tumor potential is demonstrated by their inhibition of tumor formation as compared to controls.

❖ *Treatment*

A. Traditional Cancer Treatment

Conventional cancer treatment strategies that are most commonly recommended entail physically removing the tumors, followed by chemotherapy or radiation therapy employing x-rays [33]. Surgery is the most effective treatment when the sickness is still in its early stages. Radiation therapy can damage healthy cells, tissues, and organs. Although chemotherapy has been shown to reduce morbidity and death, nearly all chemotherapeutic medicines are harmful to healthy cells, particularly fast proliferating and growing cells [34]. Drug resistance, or the phenomenon where cancer cells develop resistance to an anti-cancer medicine that initially inhibited them, is one of the primary problems associated with chemotherapy. The primary cause of this is decreased medication absorption and increased drug efflux [35]. Limitations of the conventional chemotherapeutic approach include difficult dosage selection, poor selectivity, rapid drug metabolism, and generally unfavorable side effects [36].

B. Advanced Cancer Therapies

➢ *Stem Cell Treatment*

Stem cells, which are undifferentiated cells with the potential to become any type of body cell, are found in bone marrow (BM). Stem cell therapy is one of the treatments for cancer methods that is believed to be both secure and efficient. Stem cells are currently being studied in experimental clinical studies for a variety of purposes, including the regeneration of other tissue that has been damaged. There are currently studies investigating the administration of mesenchymal stem cells (MSCs) from connective tissues, adipose tissues, and bone marrow [37].

➢ *Personalized Medication Treatment*

Targeted cancer therapy includes pharmaceuticals and other substances that are often referred to as "molecularly targeted drugs," "molecularly targeted therapies," and "precision medicines." These drugs function by interference with growth molecules, which stops the growth and spread of cancer [38].

The use of targeted therapy raised the survival percentage among individuals with advanced cancer of the pancreas from 17% to 24% when erlotinib was incorporated into standard chemotherapy. Transtuzumab, sunitinib, and imatinib, correspondingly, have revolutionized the treatment of cancers of the kidney and breast cancer, while imatinib has had a significant effect on chronic myeloid leukemia. [39].

➢ *Cancer Treatment With Ablation*

Ablation is a treatment approach that eradicates a tumor without its removal when it is under three centimeters in size and an operation is not an option. Ablation and embolization are also used in conjunction for larger tumors. However, treating tumors near major bile ducts, the diaphragm, or major blood veins may not be acceptable because this method damages some of the normal tissue surrounding the tumor. [40].

➢ *The Cryoablation Method*

Cryoablation, which includes chilling tissue to a fatal temperature and then pushing liquid to develop, is one type of ablation that seriously damages tissue. Primary malignancies, both benign and malignant, are the main indication for this treatment [41]. James Arnott found that temperatures below freezing can impact cancer cell survival when he experimented with utilizing cold temperatures to produce local numbness before surgical treatments in the eighteenth century. He increased the individual's chances of surviving by endorsing cryoablation as a worthwhile course of treatment. [42].

➢ *RFA Treatment*

Radiation coagulation (RFA) is a minimally invasive technique that destroys cancer cells by applying high-frequency electrical currents under hyperthermic conditions. It is directed by visuals. Computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound are imaging modalities used to guide needle electrodes into a tumor cell. When treating small tumors with a diameter of less than 3 cm, radiation therapy (RFA) is usually the most effective technique. Other conventional cancer treatment techniques can be used with RFA [43]. Medium tumors up to 5 cm in diameter can be cured by RFA once multiple-electrode systems or deployable devices are activated [44].

➢ *Herbal Therapy For Cancer*

Soy beans are a rich source of isoflavones, while lignans can be found in fruit, vegetables, flaxseeds, wheat, and linseeds [45, 46]. Only one of the six comparable clinical trials that have been carried out to yet has found a link between isoflavone and a lower risk of breast cancer [47]. Numerous polyphenols, such as isoflavones, are phytoestrogens that have the ability to attach to estrogen receptors and provide an estrogenic impact in the target organ or tissue.

Through research on traditional Chinese medicines (TCM), many antibreast cancer medications have been discovered; however, most of their mechanisms of action are still unknown. The six categories of TCM herbs that exhibit anti-breast cancer characteristics are: alkaloids [54,55], coumarins [52,53], flavonoids and polyphenols [50,51], terpenoids [48], quinone [49], and artesunate. [56]. High amounts of baicalin, a flavone glycoside that prevents the enzymatic production of eicosanoids, which are significant mediators of inflammation as well as the growth of prostate tumor cells, are found in *Scutellaria baicalensis.*

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The proliferation of androgen-independent PC-3 and DU145 prostate carcinoma cells in culture is inhibited by an effective concentration of the flavone baicalein, leading to an arrest of cell cycle at the G0-G1 phase [57] and apoptosis [58]. demonstrated the capacity of the oriental medicinal herb *Wedelia chinensis* (Asteraceae), which contains luteolin, apigenin, wedelolactone, and indole-3-carboxylaldehyde, to reduce androgen activity. Additionally, oral administration of W. chinensis extract slowed the proliferation of prostate cells with cancer. The anticancer action of W. chinensis extract was later shown to be attributed to three active compounds that possess the capability to disrupt the androgen receptor (AR) signaling pathway [59].

A number of substances have been investigated for their potential to prevent prostate cancer, including soy isoflavones, β-carotene, lycopenes, *Scutellaria baicalensis*, and the four active polyphenolic compounds in green tea: epicatechin, a compound known as, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG) [60]. Using specific bioassays, it has been demonstrated that EGCG in green tea can inhibit LNCaP and DU145 cells associated with prostate cancer at the G0-G1 stage of the cell cycle [61].

VII. CONCLUSION

Cancer remains a major global health challenge, but ongoing research and technological advancements continue to improve our ability to diagnose, treat, and ultimately prevent this complex group of diseases. This review summarizes on the current knowledge and highlights the recent modern therapies available providing a valuable resource for researchers engaged in the fight against cancer.

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