JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Analytical tools for examine anticancer activity –A Review

Sonia Kukreti

Assistant Professor Dev Bhoomi Uttarakhand University

Anil Singh Negi

Assistant Professor Himalayan Institute of Pharmacy & Research

Abstract-Analytical tools are necessary for both the creation of successful anticancer methods and the advancement of our knowledge of cancer. When researching anti-cancer medications, we employ a range of analytical techniques that help us gain a comprehensive grasp of the characteristics and processes related to cancer cells. This information helps us create effective treatments to fight cancer. The significance of research on anticancer drugs can be ascribed to a range of analytical methods that aid in the identification of therapeutic targets and the evaluation of drug efficacy, both of which are essential components in deepening our comprehension of the biology of cancer. Cell viability assays, clonogenic assays, flow cytometry, 2D electrophoresis, western blot caspase activation assayetc. are some of the techniques that are frequently used in cancer research that are examined in this paper. A brief overview of the principles, uses, and ways in which each analytical technique improves our knowledge of cancer is provided.

Introduction-

Cancer affects people worldwide of all ages and is one of the leading causes of death.There are several known risk factors that contribute to cancer. The impact that genetic mutations have in raising the chance of getting cancer has been well studied. In addition to genetic mutations, a number of non-genetic risk factors for cancer have also been identified, including exposure to chemicals, radiation, nutrition, infections, obesity, physical inactivity, alcohol consumption, and hormones. Cancer treatment options include chemotherapy, radiation, surgery, hormone therapy, immunotherapy, and other targeted therapies, depending on the kind of cancer and the stage of the disease. Although chemotherapy and radiation therapy can be used to treat primary cancer tumors, treating metastatic cancer is more difficult. The process of developing a cancer therapy is intricate, time-consuming, costly, and unpredictable. A cancer drug's development can cost over US$900 million and take up to 15–20 years (Neidle, 2011).

According to estimates made by the USITC (2010), only a few compounds will be approved for clinical use out of every 5000 that are examined in the initial screening stages. Drug discovery approaches have produced a significant number of chemically or naturally generated medications that can target cancer cells within the past 60 years. Of them, some have proven effective in managing and treating cancer and have been approved for clinical use, while other cancer treatments have failed in the research and development stage. Finding additional innovative anticancer medications is crucial, though, as several of the widely prescribed, well-established medications now in use have serious side effects and resistance. Synthesis of chemical compound libraries and high-throughput screening (HTS), which enables quick creation and evaluation of a large number of compounds, are two of the most advanced modern methodologies in cancer medication development (Roy, McDonald, Sittampalam, & Chaguturu, 2010). Furthermore, while creating the majority of the synthesized compounds, unexpected findings have also been produced as they have demonstrated less "drug like" qualities (Hoelder, Clarke, & Workman, 2012). Due to these problems, researchers are now looking to natural sources as an additional successful path for developing anticancer medications (Cragg & Newman, 2005; Gordaliza, 2007; Nobili et al., 2009).

The search for new natural compounds with anticancer effects is in high demand due to their chemical diversity and some of their special "drug-like" characteristics (Cragg & Newman, 2005; Gordaliza, 2007;). Three well-known naturally derived anticancer medications are paclitaxel, vincristine, and doxorubicin (Cragg, Grothaus, & Newman, 2009). Still, theDeveloping medications from natural sources has encountered many challenges.such include challenges with isolation and separation protocols, limited yield, and certain laws announced by governments of various nations onintellectual property rights (Kingston, 2010; Jachak & Saklani, 2007).

Six major biological traits (hallmarks) of cancer were proposed by Hanahan and Weinberg (2000). These traits were acquired during the development of human cancers and include sustaining proliferative signaling, inducing angiogenesis, evading growth suppressors, resisting cell death, enabling replicative immortality, and activating invasion and metastasis. Deregulated metabolism, genomic instability, immune system evasion, and inflammation were added later in 2011 as four additional hallmarks discovered throughout the development of human malignancies (Hanahan & Weinberg, 2011). Numerous in vitro methods and assays have been developed to assess each distinguishing characteristic of cancer and the choice of a specific in vitro method or test primarily depends on the research issue or questions that need to be answered. Cancer drug discovery studies frequently use in vitro assays for viability/antiproliferation (based on cellular enzymes and proteins, DNA synthesis, cellular ATP, membrane integrity and impedance), apoptosis, cell migration and invasion, angiogenesis, antioxidant and oxidative stress markers, cellular senescence, techniques to detect gene mutations and chromosomal alterations, as well as methods and techniques for gene and protein expression analysis and assays for monitoring energy metabolism in cancer cells, etc. As cutting edge methods in the search for new anticancer drugs, reporter gene assays, high content screening (HCS), and HTS have all attracted a lot of interest (Abraham, Taylor, & Haskins, 2004; Bronstein, Fortin, Stanley, Stewart, & Kricka, 1994; Fox et al., 2006).

Many side effects are associated with the cancer medications that are now on the market. Therefore, there is a great deal of scientific interest in the ongoing search for novel natural sources of anticancer drugs (A Shahiwala,2019).A variety of illnesses are included in the term "cancer," which is defined by the fast division of cells and the emergence of aberrant cells. If treatment is not received, these aberrant cells have the ability to metastasis to different organs in addition to the

surrounding tissues, which almost always results in death. According to the National Institutes of Health ("Cancer Key Facts," 2018), there will be 609640 cancer-related deaths and 1735350 new cases in the US in 2018 (Ryogo_Abu.2015).

It is crucial to choose the best in vitro assays to address the specific study question because each of the currently available assays has drawbacks of its own (Balunas & Kinghorn, 2005; Rahman et al., 2001). When choosing an in vitro assay, one should also take into account the expense, the equipment needed, and the techniques for endpoint detection (Rahman et al., 2001). Here, we list the most widely utilized in vitro methods for finding new cancer drugs.

Cell viability/cytotoxicity and anti proliferative assays-

As an in vitro test, colorimetric and fluorometric tests are used to assess cytotoxicity and preliminary anticancer efficacy for cell viability. These exams are easy to use and reasonably priced.

The primary distinction between the colorimetric and fluorometric assays is the reagent utilized in the former, which counts the total number of living cells based on mitochondrial dehydrogenase activity.

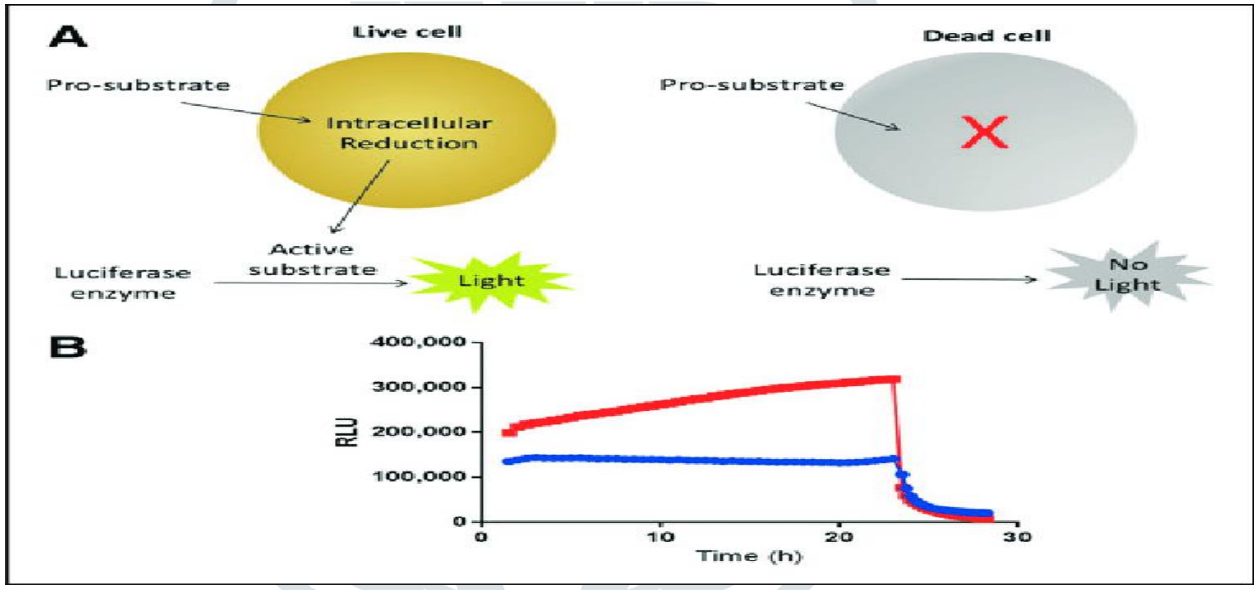

Fig 1: Cell Viability Assay

Colorimetric analysis

This test's fundamental principle is the evaluation of a molecular marker to ascertain the metabolic activity of the cell. It can be completed swiftly for a wide range of cell lines, including commercially available cells and adherent or suspended cells.

MTT Assay- 3-(4,5-Dimethylthiazol-2-yl) / 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)(3-carboxymethoxyphenyl)-5-4-(sulfophenyl)-2- Assay for 2H-tetrazolium (MTS).In the mitochondria of active cells with an OD of 570 nm, the action of mitochondrial dehydrogenases reduces tetrazolium salt to an insoluble formazan in the MTT experiment. It counts the amount of cells that are reductively active. In order to dissolve the insoluble formazan product into solution, a solubilizing solution is added in this instance.

SRB (Sulforhodamine B) assay SRB allows for the rapid, economical, and accurate testing of drug-induced cytotoxicity in adherent and suspension cell cultures. SRB is a two-sulfonic group amino xanthene dye that provides a vivid pink color. Under slightly acidic conditions in TCAfixed (trichloroacetic acid) cells, SRB attaches to the basic amino acid residues in proteins to provide a sensitive index of cellular protein. The SRB assay is also employed to evaluate colony extinction and growth. The protocol outlined here is in accordance with Kritikara and Vichai.

Technique

Fix the cells by filling each well with 10 percent (wt/vol) TCA solution (or 25 microliters if the cells are from a 96-well plate). Then, incubate the mixture at 4 degrees Celsius for one hour. Wash the plates four times with the water running gently through a plastic tube that is directly hooked to a faucet. Use paper towels to absorb any remaining water after each wash. Use a blow dryer to help dry the plates, or leave them to air dry at room temperature (20–25 ◦C). Each well needs to have 100 µL of 0.057% (wt/vol) SRB solution added.

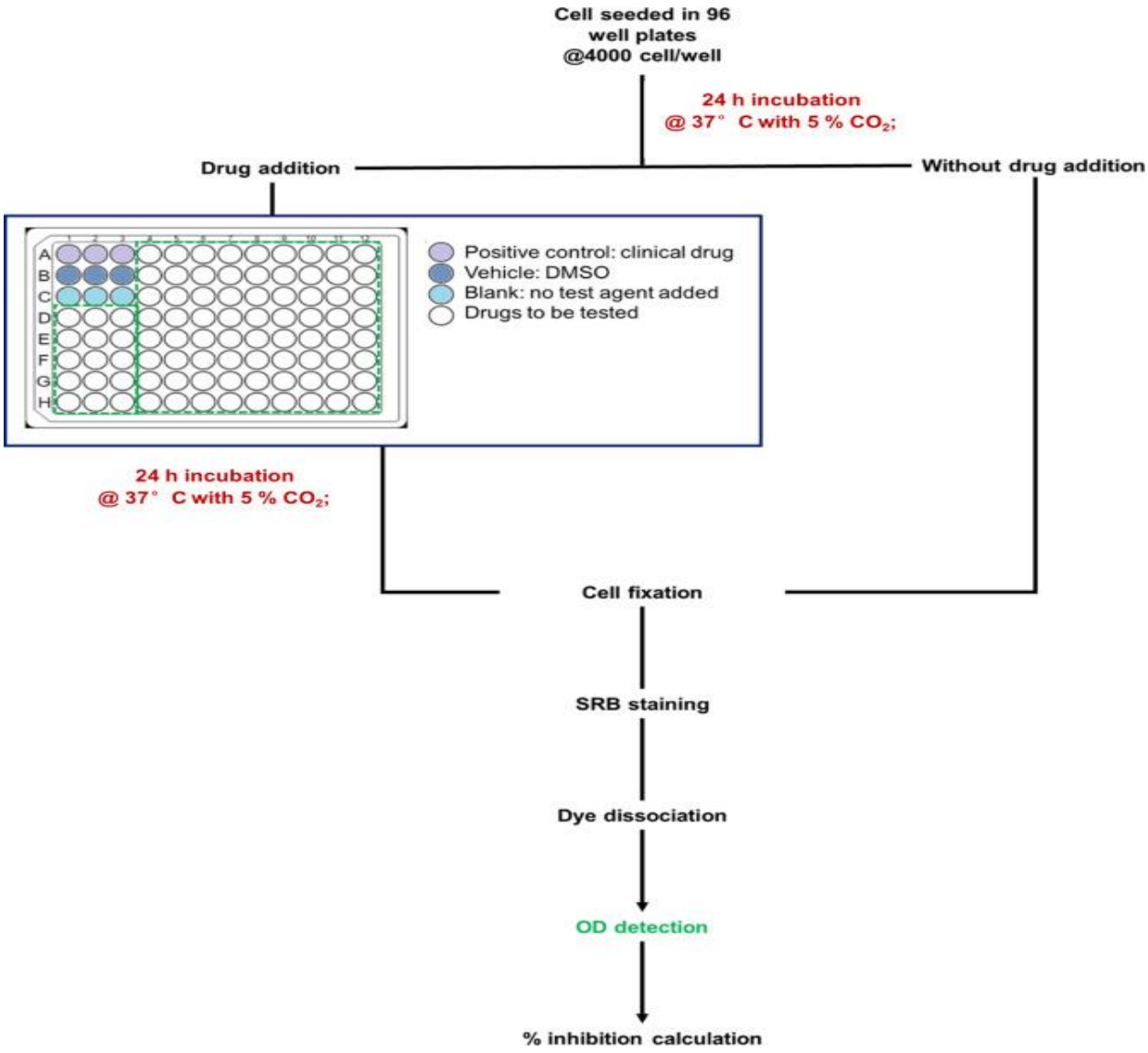

Fig 2: Steps for SRB assay

Assay for lactate dehydrogenase (LDH)

A colorimetric technique for determining cellular cytotoxicity is the lactate dehydrogenase (LDH) Cytotoxicity Assay. The stable cytosolic enzyme lactate dehydrogenase (LDH), which is released by injured cells, is quantitatively measured in this experiment. Cells have LDH in their cytoplasm. When cell viability decreases, more cell membrane leakage occurs, which releases the enzyme into the growth media. The amount of LDH released is measured using a mixed enzymatic reaction.

Iodonitrotetrazolium (INT), a tetrazolium salt, is changed into a red formazan in this process via diaphorase. This assay has been utilized in various anticancer research to evaluate cytotoxicity, apoptosis, necrosis, therapeutic efficacy, and mechanism of action (Sanjai, C., Hakkimane, S.S.,2023).

Clonogenic assays

Clonogenic tests can be used to cultivate pluripotent cells. The tumor stem-cell fraction can divide and form colonies in a semisolid matrix (agar or methylcellulose). It makes up less than 0.4% of all cells and is thought to be the most significant cell type in the development of metastases and recurrences. The tumour clonogenic assay (TCA) is widely used for assessments in drug discovery programmes and for chemosensitivity testing of tumours and xenografts. The ability of the TCA to forecast sensitivity or resistance to clinically used drugs is crucial to its utility. 62% of the comparisons for drug sensitivity and 92% of the comparisons for drug resistance were accurate when we compared the response of human tumors grown as xenografts in nude mice in the TCA in vitro to that of the clinical response(Ludwig, R.1984). When tumors were examined following serial passage in nude mice in the TCA in vitro and their reaction was compared to in vivo activity in matching xenografts, the percentage of true/false observations was found to be the same (60% and 90%, respectively). However, when the clinical response of the tumors was compared to their explants that were grown in the nude mice and treated in vivo, the highest correct predictive values were discovered. We found that 97% and 90%, respectively, of the 80 comparisons that were made correctly predicted the tumor's resistance and sensitivity. We believe that the use of TCA in well-established human tumor xenografts is crucial to the success of modern drug development initiatives. As a result, we included the TCA as a secondary assay to our strategy for finding anticancer drugs, and we discovered that several new compounds were effective; these are currently undergoing clinical trials or advanced preclinical research. As a result, the tumor clonogenic assay has demonstrated predictive significance in the chemosensitivity evaluation of both conventional and novel anticancer medications (Fiebig, H.H.2004).

Flow cytometry

Based on fluorescence signals integrated into cellular markers of proteins or DNA, flow cytometry is capable of effectively detecting and sorting cells with high sensitivity. It has been widely used to evaluate apoptosis, cell division, and the isolation of various cell types, including stem cells. Compared to other heterogeneous cells in tumors, cancer stem cells (CSCs) are frequently more resistant to cytotoxins and anticancer treatments because they serve as the seeds for tumorigenesis and metastasis. One useful method for assessing novel cancer treatment medications is to analyze CSCs while they are undergoing treatment. We present a flow cytometry approach to evaluate breast cancer stem cells (BCSCs; CD44+/CD24−/low) in human MCF-7/Dox breast cancer cells following treatment with mixed-backbone oligonucleotide inhibitors that target glucosylceramide synthase. Agents intended to target CSCs can be screened using a dependable, practical, and user-friendly method called flow cytometry analysis (Gupta, V., 2011).

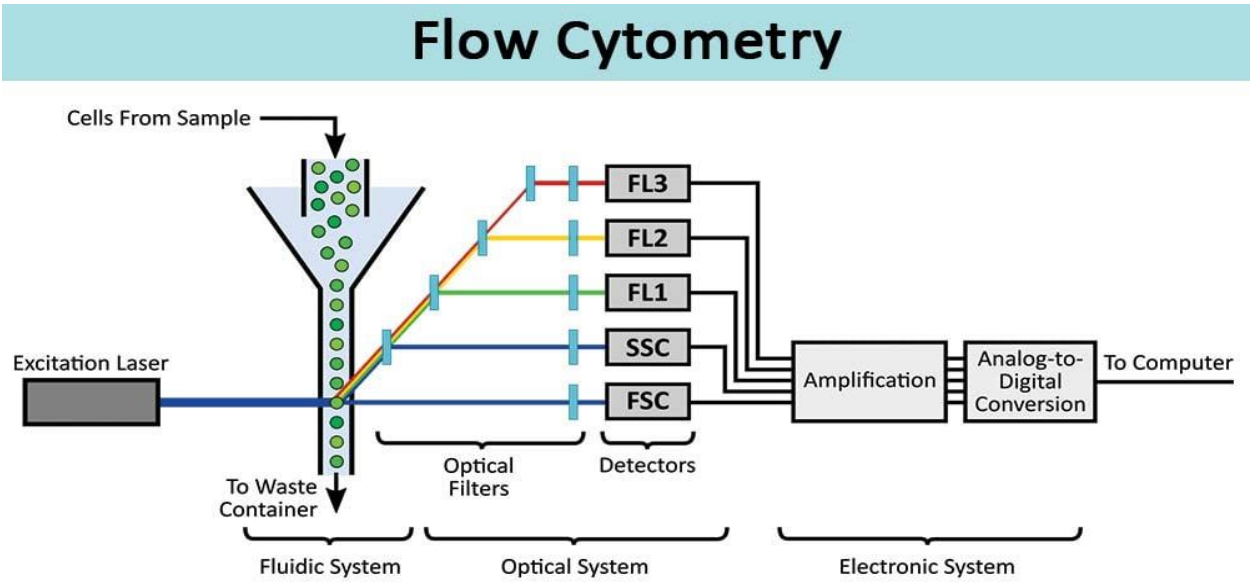

Fig 3:Flow Cytometry Steps

The uptake of the anticancer topoisomerase II toxin mitoxantrone by intact mammalian cells has been examined using flow cytometry and laser scanning confocal imaging, and the results have been connected with the generation of DNA damage. In contrast to Adriamycin, mitoxantrone exhibits very little red fluorescence when stimulated at a wavelength of 514 nm. Nonetheless, human transformed fibroblasts exposed to high quantities (5–20 µm) of mitoxantrone for one hour could show minimal levels of fluorescence utilizing flow cytometry under these excitation and emission circumstances. While drug-induced DNA-protein cross-linking demonstrated saturation, whole cell fluorescence varied with cell size and drug concentration within this dose range. Fluorescence appeared throughout the cytoplasm, nuclear membrane, and nucleoli with varying duration and dose, as demonstrated by confocal microscopy. This is understood to indicate the drug molecules' preferred locations within these tissues. In contrast to this pattern, human cells treated with Adriamycin showed strong intranuclear fluorescence. An apparent rise in chromatin-associated fluorescence was caused by the loss of the nuclear membrane during mitosis. In human cells treated for 1 hour to a low yet lethal dose (0.1 µm, generating roughly 90% cell mortality) of mitoxantrone, photon counting studies revealed a primarily cytoplasmic, likely lysosomal site for fluorescence. The work shows that high resolution investigations of the intracellular distribution of mitoxantrone in living, undamaged cells are feasible. We propose a method by which the responses of normal and multidrug-resistant cells during their effort to undergo mitosis may be influenced by the intracellular sequestration of mitoxantrone. Induction of Damage to DNA in Human Fibroblasts that Have Transformed. In cells treated with modest concentrations (0.16-2.5 MM) of mi toxantrone, drug-induced DNA strand breaking (including breakage resulting from trapped cleavable complexes) could be identified, with evidence of saturation of lesion frequencies at drug concentrations between 1.25 and 2.5 MM The X-ray calibrations (refer to "Materials and Methods") revealed damage comparable to roughly 10 Gy at 2.5 MMmitox antrone; nonetheless, the measured levels of strand breakage were within the upper limits of the denaturation assay. comparable increases in DNA-protein cross-linking levels were elicited at low mitoxantrone doses, with evidence of saturation within a comparable 2-4 MMdose range, . By computer fitting (35) the combined clonogenic survival data from three studies, the assessment of mitoxantrone sensitivity in parallel clonogenic assays using the previously described approach revealed a DOvalue of 27.3 nM mitoxantrone (1 h drug exposure) (data not shown). For MRC5CVI cells, the 10% survival dose fell between 80 and 100 nM mito xantrone. Nuclear membrane, nucleolar, and general cytoplasmic staining appeared in a dose-dependent manner, with orescence predominantly seen within cytoplasmic inclusions. This pattern contrasted sharply with that seen in cells treated with Adriamycin where the nuclear membrane and perinucleolar regions of the nucleus showed strong staining. When cells subjected to 1 MM drug doses were analyzed using images, it was shown that cells treated with mitoxantrone had nuclear fluorescence levels that were >4 times lower than those treated with

Adriamycin. The pattern of mitoxantrone uptake was ascertained by adjusting the incubation period while maintaining a constant drug dose of 20 MM. Images of MRC5CVI cells after 15 minutes, 1 hour, and 2 hours of incubation are displayed in Fig. 4. With the incubation duration, the fluorescence intensity rose and stabilized at about one hour. After fifteen minutes, fluorescent material was visible inside cytoplasmic inclusions that tended to cluster close to the nucleus. Nuclear membranes and nucleolar-like entities were well-defined, and the fluorescence intensity of nuclear-associated structures grew preferentially during the course of the incubation period. By sectioning at around two MHIintervals, a Z-axis series representing the fluorescence distribution of mitox antrone in MRC5CVI cells was produced .Z-sections next to the attachment surface. The intracellular distribution mentioned before was validated by the subsequent photos. A mitotic cell is one of the sections depicted in Fig. 5, where the condensed chromatids are uniformly stained and the highly fluorescent cytoplasmic inclusions are less noticeable (Smith, P.J., Sykes,1992).

2D electrophoresis-

Proteomics technologies provide a means of tracking the pattern of protein expression in tumor cells, which may lead to the identification of novel biomarkers for cancer early diagnosis and detection. In order to gain a better understanding of the molecular basis of cancer pathogenesis and the validation and characterization of disease-associated proteins, a variety of proteomic tools, including 2D-PAGE, 2D-DIGE, SELDI-ToF-MS technology, protein arrays, ICAT, iTRAQ, and MudPIT, have been used for differential analysis of biological samples, including cell lysates, cell secretome (conditioned medium), serum, plasma, tumour tissue, and nipple aspirate fluid. The number of papers on the discovery of new cancer biomarkers has increased significantly in recent years. As a result, the contribution of proteomics technologies to serum and conditioned medium-based oncology research, notably for lung cancer, has been the main focus of this study. While there are numerous efficient treatments available for early.Despite identification and diagnosis, it continues to be a leading cause of illness and death. The complicated nature of cancer shows both protein and genetic alterations that occur within a cell. Carcinoembryonic antigen (CEA), the first protein cancer marker, was discovered in patient serum in 1965 and was used to diagnose colorectal cancer. Prostate-specific antigen (PSA) for prostate cancer, CA-19 for colorectal and pancreatic cancer, CA-15-3 for breast cancer, and CA-125 for ovarian cancer are among the other biomarkers identified in the 1970s and 1980s. All biomarkers, however, are not useful in every clinical setting. For instance, PSA is widely used in clinical practice; however, since not all patients with increased PSA levels have aggressive prostate cancer, about one-third of those people frequently have unnecessary medical procedures performed on them. There are no reliable biomarkers for lung cancer or melanoma that can be used to test for the disease in its early stages. Finding a new tumor (Maurya, P.,2007).

Western blot caspase activation assay

SGC-7901 and MKN-45 cells (1×105 cells/well), seeded in 10 cm culture dishes, were cultured for 24 hours, and then treated with 0 μg/mL (control), 40 μg/mL, 80 μg/mL, or 120 μg/mL GS-Rd for 48 hours. The cells were lysed in PMSF-containing RIPA buffer in preparation for western blotting. PVDF membranes were coated with the protein after it had been separated via polyacrylamide gel electrophoresis with 12% sodium dodecyl sulfate (SDS-PAGE). Before applying the primary antibodies, which included β-actin (1:1000; Cell Signaling Technology, USA), Bax (1:1000; Cell Signaling Technology, USA), Bcl-2 (1:1000; Cell Signaling Technology, USA), Caspase-3 (1:1000; Cell Signaling Technology, USA), and Caspase-9 (1:1000; Cell Signaling Technology, USA), Cyclin D1 (1:1000; Seeded in 10 cm culture dishes,

SGC-7901 and MKN-45 cells. Cell Signaling Technology, USA), were incubated for an overnight period. Blots were cleaned and then treated with secondary antibodies. In the Human GC cells of each group, the Caspase-9 and Caspase-3 protein quantities were found. Additionally, the protein concentrations of Bcl-2 and Bax were measured (Tian, Y.Z,2020).

Reference-

1. Balasubramanian, S., Hurley, L.H. and Neidle, S., 2011. Targeting G-quadruplexes in gene promoters: a novel anticancer strategy?. *Nature reviews Drug discovery*, *10*(4), pp.261-275.
2. Roy, A., R McDonald, P., Sittampalam, S. and Chaguturu, R., 2010. Open access high throughput drug discovery in the public domain: a Mount Everest in the making. *Current pharmaceutical biotechnology*, *11*(7), pp.764-778.
3. Hoelder, S., Clarke, P.A. and Workman, P., 2012. Discovery of small molecule cancer drugs: successes, challenges and opportunities. *Molecular oncology*, *6*(2), pp.155-176.
4. Baharuddin, A.A., Roosli, R.A., Zakaria, Z.A. and Tohid, S.F.M., 2021. Dicranopteris linearis A potential medicinal plant with anticancer properties. *Boletín Latinoam. Y Del Caribe De Plantas Med. Y Aromáticas*, *20*, pp.28-37.
5. Cragg, G.M., Grothaus, P.G. and Newman, D.J., 2009. Impact of natural products on developing new anti-cancer agents. *Chemical reviews*, *109*(7), pp.3012-3043.
6. Amirkia, V. and Heinrich, M., 2015. Natural products and drug discovery: A survey of stakeholders in industry and academia. *Frontiers in pharmacology*, *6*, p.237.
7. Hanahan, D. and Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *cell*, *144*(5), pp.646-674.
8. Ediriweera, M.K., Tennekoon, K.H. and Samarakoon, S.R., 2019. In vitro assays and techniques utilized in anticancer drug discovery. *Journal of Applied Toxicology*, *39*(1), pp.38-71.
9. Shahiwala, A., Shehab, N.G., Khider, M. and Khan, R., 2019. Chitosan nanoparticles as a carrier for indigofera intricata plant extract: Preparation, characterization and anticancer activity. *Current Cancer Therapy Reviews*, *15*(2), pp.162-169.
10. Sanjai, C., Hakkimane, S.S., Guru, B.R. and Gaonkar, S.L., 2023. A comprehensive review on anticancer evaluation techniques. *Bioorganic Chemistry*, p.106973.
11. Khan, T., Date, A., Chawda, H. and Patel, K., 2019. Polysaccharides as potential anticancer agents—A review of their progress. *Carbohydrate polymers*, *210*, pp.412-428.
12. Hamedeyazdan, S., Fathiazad, F., Sharifi, S. and Nazemiyeh, H., 2012. Antiproliferative activity of Marrubium persicum extract in the MCF-7 human breast cancer cell line. *Asian Pacific Journal of Cancer Prevention*, *13*(11), pp.5843-5848.
13. Fiebig, H.H., Maier, A. and Burger, A.M., 2004. Clonogenic assay with established human tumour xenografts: correlation of in vitro to in vivo activity as a basis for anticancer drug discovery. *European journal of cancer*, *40*(6), pp.802-820.

14. Chakraborti, D., Mukherjee, S.C., Pati, S., Sengupta, M.K., Rahman, M.M., Chowdhury, U.K., Lodh, D., Chanda, C.R., Chakraborti, A.K. and Basu, G.K., 2003. Arsenic groundwater contamination in Middle Ganga Plain, Bihar, India: a future danger?. *Environmental health perspectives*, *111*(9), pp.1194-1201.
15. Franken, N.A., Rodermond, H.M., Stap, J., Haveman, J. and Van Bree, C., 2006. Clonogenic assay of cells in vitro. *Nature protocols*, *1*(5), pp.2315-2319.
16. Plumb, J.A., 2004. Cell sensitivity assays: clonogenic assay. *Cancer cell culture: methods and protocols*, pp.159-164.
17. Gupta, V., Zhang, Q.J. and Liu, Y.Y., 2011. Evaluation of anticancer agents using flow cytometry analysis of cancer stem cells. *Drug Design and Discovery: Methods and Protocols*, pp.179-191.
18. Smith, P.J., Sykes, H.R., Fox, M.E. and Furlong, I.J., 1992. Subcellular distribution of the anticancer drug mitoxantrone in human and drug-resistant murine cells analyzed by flow cytometry and confocal microscopy and its relationship to the induction of DNA damage. *Cancer research*, *52*(14), pp.4000-4008.
19. Ludwig, R., Alberts, D.S., Miller, T.P. and Salmon, S.E., 1984. Evaluation of anticancer drug schedule dependency using an in vitro human tumor clonogenic assay. *Cancer chemotherapy and pharmacology*, *12*, pp.135-141.
20. Maurya, P., Meleady, P., Dowling, P. and Clynes, M., 2007. Proteomic approaches for serum biomarker discovery in cancer. *Anticancer research*, *27*(3A), pp.1247-1255.
21. Tian, Y.Z., Liu, Y.P., Tian, S.C., Ge, S.Y., Wu, Y.J. and Zhang, B.L., 2020. Antitumor activity of ginsenoside Rd in gastric cancer via up-regulation of Caspase-3 and Caspase-9. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *75*(4), pp.147-150.