INVITRO CYTOTOXIC AND CYTOPROTECTIVE ACTIVITY OF SILIBININ AND GENISTEIN ON BREAST AND COLON CELL LINES.

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"Invitro Cytotoxic And Cytoprotective Activity of Silibinin And Genistein On Breast And Colon Cell Lines."** submitted by the student bearing **Reg. No:26103095** to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment for the award of degree of **MASTER OF PHARMACY** in **PHARMACOLOGY** was evaluated by us during the examination held on…….

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This is to certify that the work embodied in this dissertation "**Invitro Cytotoxic And Cytoprotective Activity of Silibinin And Genistein On Breast And Colon Cell Lines.",** submitted to "The Tamil Nadu Dr.M.G.R. Medical University", Chennai, was carried out by **Mr.Deshmukh Sameer Bhalchandra [Reg.No: 26103095],** for the Partial fulfillment of degree of **MASTER OF PHARMACY** in Department Of Pharmacology under direct supervision of **Mr. V. Rajesh , M.Pharm.,** Professor & Head**,** Department Of Pharmacology, J.K.K.Nattaraja College of Pharmacy, Komarapalayam, during the academic year 2011-2012.

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DECLARATION

The work presented in this dissertation entitled **"Invitro Cytotoxic And Cytoprotective Activity of Silibinin And Genistein On Breast And Colon Cell Lines.",** was carried out by me, under the direct supervision of **Mr. V. Rajesh, M.Pharm.,** Professor & Head, Department Of Pharmacology, J.K.K.Nattaraja College of Pharmacy, Komarapalayam.

I further declare that, this work is original and has not been submitted in part or full for the award of any other degree or diploma in any other university.

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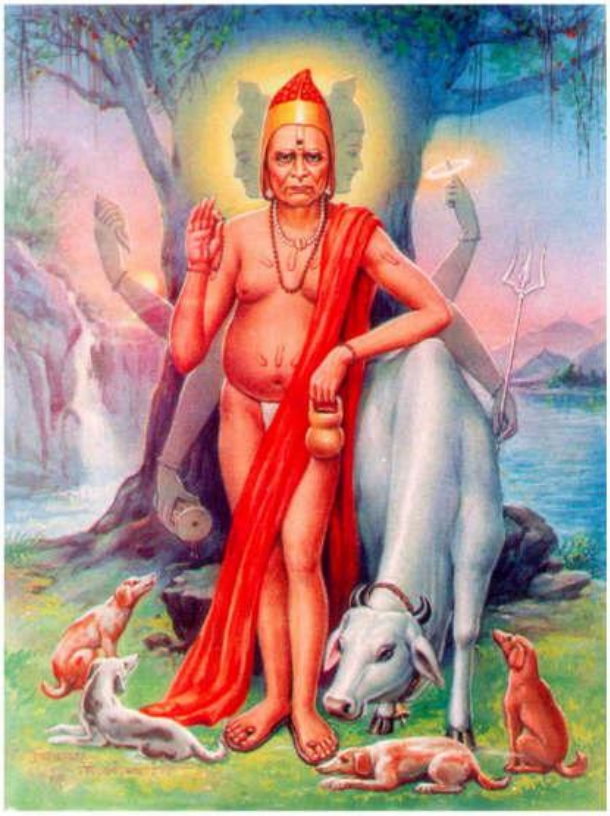
“DEDICATED TO MY GURU, MY BELOVED PARENTS AND MY FRIENDS”



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INTRODUCTION

Cancer known medically as a malignant neoplasm, is a large group of different diseases, all involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body. The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. Not all tumors are cancerous. Benign tumors do not grow uncontrollably, do not invade neighboring tissues, and do not spread throughout the body.

Determining what causes cancer is complex. Many things are known to increase the risk of cancer, including tobacco use, certain infections, radiation, lack of physical activity, poor diet and obesity, and environmental pollutants. These can directly damage genes or combine with existing genetic faults within cells to cause the disease. Approximately five to ten percent of cancers are entirely hereditary.

Cancer can be detected in a number of ways, including the presence of certain signs and symptoms, screening tests, or medical imaging. Once a possible cancer is detected it is diagnosed by microscopic examination of a tissue sample. Cancer is usually treated with chemotherapy, radiation therapy and surgery. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age. In 2007, cancer caused about 13% of all human deaths worldwide (7.9 million). Rates are rising as more people live to an old age and as mass lifestyle changes occur in the developing world.

HISTORY

The earliest written record regarding cancer is from 3000 BC in the Egyptian Edwin Smith Papyrus and describes cancer of the breast. Cancer however has existed for all of human history. Hippocrates (ca. 460 BC – ca. 370 BC) described several kinds of cancer, referring to them with the Greek word *carcinos* (crab or crayfish). This name comes from the appearance of the cut surface of a solid malignant tumour, with "the veins stretched on all sides as the animal the crab has its feet, whence it derives its name". The Greek, Celsus (ca. 25 BC - 50 AD) translated *carcinos* into the Latin *cancer*, also meaning crab and recommended surgery as treatment. Galen (2nd century AD) disagreed with the use of surgery and recommended purgatives instead. These recommendations largely stood for 1000 years.

In the 15th, 16th and 17th centuries, it became more acceptable for doctors to dissect bodies to discover the cause of death. The German professor Wilhelm Fabry believed that breast cancer was caused by a milk clot in a mammary duct. The Dutch professor Francois de la Boe Sylvius, a follower of Descartes, believed that all disease was the outcome of chemical processes, and that acidic lymph fluid was the cause of cancer. His contemporary Nicolaes Tulp believed that cancer was a poison that slowly spreads, and concluded that it was contagious.

The physician John Hill described tobacco snuff as the cause of nose cancer in 1761. This was followed by the report in 1775 by British surgeon Percivall Pott that cancer of the scrotum was a common disease among chimney sweeps. With the widespread use of the microscope in the 18th century, it was discovered that the 'cancer poison' spread from the primary tumor through the lymph nodes to other sites ("metastasis"). This view of the disease was first formulated by the English surgeon Campbell De Morgan between 1871 and 1874.

SIGNS AND SYMPTOMS

When cancer begins it invariably produces no symptoms with signs and symptoms only appearing as the mass continue to grow ulcerates. The findings that result depend on the type and location of the cancer. Few symptoms are specific, with many of them also frequently occurring in individuals who have other conditions. Cancer is the new "great imitator". Thus it is not uncommon for people diagnosed with cancer to have been treated for other diseases to which it was assumed their symptoms were due.

LOCAL EFFECTS

Local symptoms may occur due to the mass of the tumor or its ulceration. For example mass effects from lung cancer can cause blockage of the bronchus resulting in cough or pneumonia, esophageal cancer can cause narrowing of the esophagus making it difficult or painful to swallow, and colorectal cancer may lead to narrowing or blockages in the bowel resulting in changes in bowel habits. Masses of breast or testicles may be easily felt. Ulceration can cause bleeding which, if it occurs in the lung, will lead to coughing up blood, in the bowels to anemia or rectal bleeding, in the bladder to blood in the urine, and in the uterus to vaginal bleeding. Although localized pain may occurs in advanced cancer, the initial swelling is usually painless. Some cancers can cause build up of fluid within the chest or abdomen.

SYSTEMIC SYMPTOMS

General symptoms occur due to distant effects of the cancer that are not related to direct or metastatic spread. These may include: unintentional weight loss, fever, being excessively tired, and changes to the skin. Hodgkin disease, leukemias, and cancers of the liver or kidney can cause a persistent fever of unknown origin.

Specific constellations of systemic symptoms, termed paraneoplastic phenomenone, may occur with some cancers. Examples include the appearance of myasthenia gravis in thymoma and clubbing in lung cancer.

METASTASIS

Symptoms of metastasis are due to the spread of cancer to other locations in the body. They can include enlarged lymph nodes (which can be felt or sometimes seen under the skin and are typically hard), hepatomegaly (enlarged liver) or splenomegaly (enlarged spleen) which can be felt in the abdomen, pain or fracture of affected bones, and neurological symptoms. **(Moscow *et al.*, 2007**)

CAUSES

Cancers are primarily an environmental disease with 90-95% of cases attributed to environmental factors and 5-10% due to genetics. *Environmental*, as used by cancer researchers, means any cause that is not genetic, not merely pollution. Common environmental factors that contribute to cancer death include tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity, and environmental pollutants.

It is nearly impossible to prove what caused a cancer in any individual, because most cancers have multiple possible causes. For example, if a person who uses tobacco heavily develops lung cancer, then it was probably caused by the tobacco use, but since everyone has a small chance of developing lung cancer as a result of air pollution or radiation, then there is a small chance that the cancer developed because of air pollution or radiation.

CHEMICALS

Cancer pathogenesis is traceable back to DNA mutations that impact cell growth and metastasis. Substances that cause DNA mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens. Particular substances have been linked to specific types of cancer. Tobacco smoking is associated with many forms of cancer, and causes 90% of lung cancer.

Many mutagens are also carcinogens, but some carcinogens are not mutagens. Alcohol is an example of a chemical carcinogen that is not a mutagen. In Western Europe 10% of cancers in males and 3% of cancers in females are attributed to alcohol.

Decades of research has demonstrated the link between tobacco use and cancer in the lung, larynx, head, neck, stomach, bladder, kidney, esophagus and pancreas. Tobacco smoke contains over fifty known carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons. Tobacco is responsible for about one in three of all cancer deaths in the developed world, and about one in five worldwide. Lung cancer death rates in the United States have mirrored smoking patterns, with increases in smoking followed by dramatic

increases in lung cancer death rates and, more recently, decreases in smoking rates since the 1950s followed by decreases in lung cancer death rates in men since 1990. However, the numbers of smokers worldwide is still rising, leading to what some organizations have described as the *tobacco epidemic*.

Cancer related to one's occupation is believed to represent between 2–20% of all cases. Every year, at least 200,000 people die worldwide from cancer related to their workplace. Most cancer deaths caused by occupational risk factors occur in the developed world. It is estimated that approximately 20,000 cancer deaths and 40,000 new cases of cancer each year in the U.S. are attributable to occupation. Millions of workers run the risk of developing cancers such as lung cancer and mesothelioma from inhaling asbestos fibers and tobacco smoke, or leukemia from exposure to benzene at their workplaces.

DIET

Diet, physical inactivity, and obesity are related to approximately 30–35% of cancer cases. In the United States excess body weight is associated with the development of many types of cancer and is a factor in 14–20% of all cancer deaths. Physical inactivity is believed to contribute to cancer risk not only through its effect on body weight but also through negative effects on immune system and endocrine system.

Diets that are low in vegetables, fruits and whole grains, and high in processed or red meats are linked with a number of cancers. A high salt diet is linked to gastric cancer, aflatoxin B1, a frequent food contaminate, with liver cancer, and Betel nut chewing with oral cancer. This may partly explain differences in cancer incidence in different countries for example gastric cancer is more common in Japan with its high salt diet and colon cancer is more common in the United States. Immigrants develop the risk of their new country, often within one generation, suggesting a substantial link between diet and cancer.

INFECTION

Worldwide approximately 18% of cancers are related to infectious diseases. This proportion varies in different regions of the world from a high of 25% in Africa to less than 10% in the developed world. Viruses are the usual infectious agents that cause cancer but bacteria and parasites may also have an effect.

A virus that can cause cancer is called an *oncovirus*. These include human papillomavirus (cervical carcinoma), Epstein-Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma), Kaposi's sarcoma herpesvirus (Kaposi's Sarcoma and primary effusion lymphomas), hepatitis B and hepatitis C viruses (hepatocellular carcinoma), and Human T-cell leukemia virus-1 (T-cell leukemias). Bacterial infection may also increase the risk of cancer, as seen in Helicobacter pylori-induced gastric carcinoma. Parasitic infections strongly associated with cancer include *Schistosoma haematobium* (squamous cell carcinoma of the bladder) and the liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*(cholangiocarcinoma).

RADIATION

Up to 10% of invasive cancers are related to radiation exposure, including both ionizing radiation and non-ionizing radiation. Additionally, the vast majority of non-invasive cancers are non-melanoma skin cancers caused by non-ionizing ultraviolet radiation.

Sources of ionizing radiation include medical imaging, and radon gas. Radiation can cause cancer in most parts of the body, in all animals, and at any age, although radiation-induced solid tumors usually take 10–15 years, and can take up to 40 years, to become clinically manifest, and radiation-induced leukemias typically require 2–10 years to appear. Some people, such as those with nevoid basal cell carcinoma syndrome or retinoblastoma, are more susceptible than average to developing cancer from radiation exposure. Children and adolescents are twice as likely to develop radiation-induced leukemia as adults; radiation exposure before birth has ten times the effect. Ionizing radiation is not a particularly strong mutagen. Residential exposure to radon gas, for example, has similar cancer risks as passive smoking. Low-dose exposures, such as living near a nuclear power plant, are

generally believed to have no or very little effect on cancer development. Radiation is a more potent source of cancer when it is combined with other cancer-causing agents, such as radon gas exposure plus smoking tobacco.

Unlike chemical or physical triggers for cancer, ionizing radiation hits molecules within cells randomly. If it happens to strike a chromosome, it can break the chromosome, result in an abnormal number of chromosomes, inactivate one or more genes in the part of the chromosome that it hit, delete parts of the DNA sequence, cause chromosome translocations, or cause other types of chromosome abnormalities. Major damage normally results in the cell dying, but smaller damage may leave a stable, partly functional cell that may be capable of proliferating and developing into cancer, especially if tumor suppressor genes were damaged by the radiation. Three independent stages appear to be involved in the creation of cancer with ionizing radiation: morphological changes to the cell, acquiring cellular immortality (losing normal, life-limiting cell regulatory processes), and adaptations that favor formation of a tumor. Even if the radiation particle does not strike the DNA directly, it triggers responses from cells that indirectly increase the likelihood of mutations.

Medical use of ionizing radiation is a growing source of radiation-induced cancers. Ionizing radiation may be used to treat other cancers, but this may, in some cases, induce a second form of cancer. It is also used in some kinds of medical imaging. One report estimates that approximately 29,000 future cancers could be related to the approximately 70 million CT scans performed in the US in 2007. It is estimated that 0.4% of cancers in 2007 in the United States are due to CTs performed in the past and that this may increase to as high as 1.5–2% with rates of CT usage during this same time period.

Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies. Clear evidence establishes ultraviolet radiation, especially the non-ionizing medium wave UVB, as the cause of most non-melanoma skin cancers, which are the most common forms of cancer in the world.

HEREDITY

The vast majority of cancers are non-hereditary ("sporadic cancers"). Hereditary cancers are primarily caused by an inherited genetic defect. Less than 0.3% of the population carriers of a genetic mutation which has a large effect on cancer risk and these cause less than 3–10% of all cancer. Some of these syndromes include: certain inherited mutations in the genes *BRCA1* and *BRCA2* with a more than 75% risk of breast cancer and ovarian cancer, and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) which is present in about 3% of people with colorectal cancer, among others.

PHYSICAL AGENTS

Some substances cause cancer primarily through their physical, rather than chemical, effects on cells.

A prominent example of this is prolonged exposure to asbestos, naturally occurring mineral fibers which are a major cause of mesothelioma, a type of lung cancer. Other substances in this category, including both naturally occurring and synthetic asbestos-like fibers such as wollastonite, attapulgite, glass wool, and rock wool, are believed to have similar effects.

Nonfibrous particulate materials that cause cancer include powdered metallic cobalt and nickel, and crystalline silica (quartz, cristobalite, and tridymite).

Usually, physical carcinogens must get inside the body (such as through inhaling tiny pieces) and require years of exposure to develop cancer.

Physical trauma resulting in cancer is relatively rare. Claims that breaking bone resulted in bone cancer, for example, have never been proven. Similarly, physical trauma is not accepted as a cause for cervical cancer, breast cancer, or brain cancer.

One accepted source is frequent, long-term application of hot objects to the body. It is possible that repeated burns on the same part of the body, such as those produced by kanger and kairo heaters (charcoal hand warmers), may produce skin cancer, especially if carcinogenic chemicals are also present. Frequently drinking scalding hot tea may produce esophageal cancer.

Generally, it is believed that the cancer arises, or a pre-existing cancer is encouraged, during the process of repairing the trauma, rather than the cancer being caused directly by the trauma. However, repeated injuries to the same tissues might promote excessive cell proliferation, which could then increase the odds of a cancerous mutation. There is no evidence that inflammation itself causes cancer.

HORMONES

Some hormones play a role in the development of cancer by promoting cell proliferation. Hormones are important agents in sex-related cancers such as cancer of the breast, endometrial, prostate, ovary, and testis, and also of thyroid cancer and bone cancer.

An individual's hormone levels are mostly determined genetically, so this may at least partly explains the presence of some cancers that run in families that do not seem to have any cancer-causing genes. For example, the daughters of women who have breast cancer have significantly higher levels of estrogen and progesterone than the daughters of women without breast cancer. These higher hormone levels may explain why these women have higher risk of breast cancer, even in the absence of a breast-cancer gene. Similarly, men of African ancestry have significantly higher levels of testosterone than men of European ancestry, and have a correspondingly much higher level of prostate cancer. Men of Asian ancestry, with the lowest levels of testosterone-activating androstanediol glucuronide, have the lowest levels of prostate cancer.

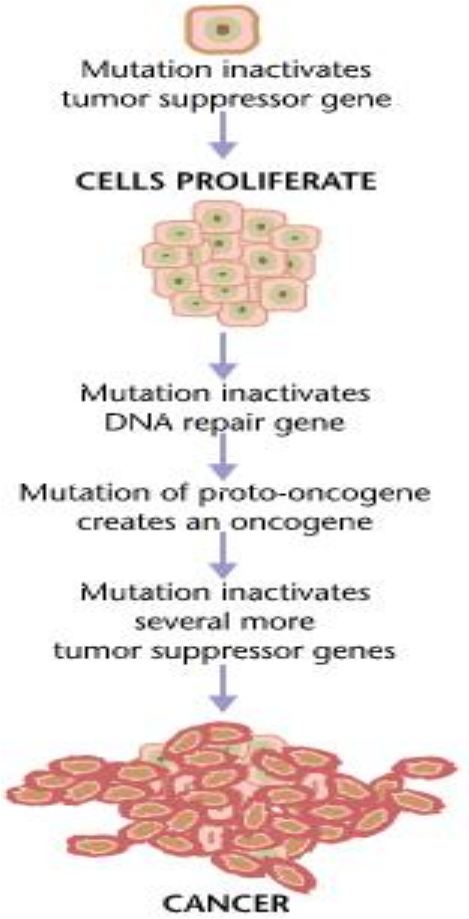
However, non-genetic factors are also relevant: obese people have higher levels of some hormones associated with cancer and a higher rate of those cancers. Women who take hormone replacement therapy have a higher risk of developing cancers associated with those hormones. On the other hand, people who exercise far more than average have lower levels of these hormones, and lower risk of cancer. Osteosarcoma may be promoted by growth hormones. Some treatments and prevention approaches leverage this cause by artificially reducing hormone levels, and thus discouraging hormone-sensitive cancers. (**Kinzler *et al.*, 2002)**

OTHER

Excepting the rare transmissions that occur with pregnancies and only a marginal few organ donors, cancer is generally not a transmissible disease. The main reason for this is tissue graft rejection caused by MHC incompatibility. In humans and other vertebrates, the immune system uses MHC antigens to differentiate between "self" and "non-self" cells because these antigens are different from person to person. When non-self antigens are encountered, the immune system reacts against the appropriate cell. Such reactions may protect against tumour cell engraftment by eliminating implanted cells. In the United States, approximately 3,500 pregnant women have a malignancy annually, and transplacental transmission of acute leukaemia, lymphoma, melanoma and carcinoma from mother to fetus has been observed. The development of donor-derived tumors from organ transplants is exceedingly rare. The main cause of organ transplant associated tumors seems to be malignant melanoma, that was undetected at the time of organ harvest. Cancer from one organism will usually grow in another organism of that species, as long as they share the same histocompatibility genes, proven using mice; however this would never happen in a real-world setting except as described above.

In non-humans, a few types of transmissible cancer have been described, wherein the cancer spreads between animals by transmission of the tumor cells themselves. This phenomenon is seen in dogs with Sticker's sarcoma, also known as canine transmissible venereal tumor, as well as devil facial tumour disease in Tasmanian devils.

PATHOPHYSIOLOGY



Cancer is fundamentally a disease of failure of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, the genes which regulate cell growth and differentiation must be altered.

The affected genes are divided into two broad categories. Oncogenes are genes which promote cell growth and reproduction. Tumor suppressor genes are genes which inhibit cell division and survival. Malignant transformation can occur through the formation of novel oncogenes, the inappropriate over-expression of normal oncogenes, or by the under-expression or disabling of tumor suppressor genes. Typically, changes in *many* genes are required to transform a normal cell into a cancer cell.

Genetic changes can occur at different levels and by different mechanisms. The gain or loss of an entire chromosome can occur through errors in mitosis. More common are mutations, which are changes in the nucleotide sequence of genomic DNA.

Large-scale mutations involve the deletion or gain of a portion of a chromosome. Genomic amplification occurs when a cell gains many copies (often 20 or more) of a small chromosomal locus, usually containing one or more oncogenes and adjacent genetic material. Translocation occurs when two separate chromosomal regions become abnormally fused, often at a characteristic location. A well-known example of this is the Philadelphia chromosome, or translocation of chromosomes 9 and 22, which occurs in chronic myelogenous leukemia, and results in production of the BCR-abl fusion protein, an oncogenic tyrosine kinase.

Small-scale mutations include point mutations, deletions, and insertions, which may occur in the promoter region of a gene and affect its expression, or may occur in the gene's coding sequence and alter the function or stability of its protein product. Disruption of a single gene may also result from integration of genomic material from a DNA virus or retrovirus, and resulting in the expression of *viral* oncogenes in the affected cell and its descendants.

Replication of the enormous amount of data contained within the DNA of living cells will probabilistically result in some errors (mutations). Complex error correction and prevention is built into the process, and safeguards the cell against cancer. If significant error occurs, the damaged cell can "self-destruct" through programmed cell death, termed apoptosis. If the error control processes fail, then the mutations will survive and be passed along to daughter cells.

Some environments make errors more likely to arise and propagate. Such environments can include the presence of disruptive substances called carcinogens, repeated physical injury, heat, ionising radiation, or hypoxia. . **(Kim *et al.*, 2006)**

The errors which cause cancer are *self-amplifying* and *compounding*, for example:

- A mutation in the error-correcting machinery of a cell might cause that cell and its children to accumulate errors more rapidly.
- A further mutation in an oncogene might cause the cell to reproduce more rapidly and more frequently than its normal counterparts.
- A further mutation may cause loss of a tumour suppressor gene, disrupting the apoptosis signaling pathway and resulting in the cell becoming immortal.
- A further mutation in signaling machinery of the cell might send error-causing signals to nearby cells.

The transformation of normal cell into cancer is akin to a chain reaction caused by initial errors, which compound into more severe errors, each progressively allowing the cell to escape the controls that limit normal tissue growth. This rebellion-like scenario becomes an undesirable survival of the fittest, where the driving forces of evolution work against the body's design and enforcement of order. Once cancer has begun to develop, this ongoing process, termed *clonal evolution* drives progression towards more invasive stages. (**Croce, 2008**)

DIAGNOSIS

Most cancers are initially recognized either because of the appearance of signs or symptoms or through screening. Neither of these lead to a definitive diagnosis, which requires the examination of a tissue sample by a pathologist. People with suspected cancer are investigated with medical tests. These commonly include blood tests, X-rays, CT scans and endoscopy.

CLASSIFICATION

Cancers are classified by the type of cell that the tumor cells resemble and therefore presumed to be the origin of the tumor. These types include:

- Carcinoma: Cancers derived from epithelial cells. This group includes many of the most common cancers, particularly in the aged, and include nearly all those developing in the breast, prostate, lung, pancreas, and colon.
- Sarcoma: Cancers arising from connective tissue (i.e. bone, cartilage, fat, nerve), each of which develop from cells originating in mesenchymal cells outside the bone marrow.
- Lymphoma and leukemia: These two classes of cancer arise from hematopoietic (blood-forming) cells that leave the marrow and tend to mature in the lymph nodes and blood, respectively.
- Germ cell tumor: Cancers derived from pluripotent cells, most often presenting in the testicle or the ovary (seminoma and dysgerminoma, respectively).
- Blastoma: Cancers derived from immature "precursor" cells or embryonic tissue. These are also most common in children. (**Kinzler *et al.*, 2002)**

Cancers are usually named using *-carcinoma*, *-sarcoma* or *-blastoma* as a suffix, with the Latin or Greek word for the organ or tissue of origin as the root. For example, cancers of the liver parenchyma arising from malignant epithelial cells is called *hepatocarcinoma*, while a malignancy arising from primitive liver precursor cells is called a hepatoblastoma, and a cancer arising from fat cells is called a *liposarcoma*.

For some common cancers, the English organ name is used. For example, the most common type of breast cancer is called *ductal carcinoma of the breast*. Here, the adjective *ductal* refers to the appearance of the cancer under the microscope, which suggests that it has originated in the milk ducts.

Benign tumors (which are not cancers) are named using *-oma* as a suffix with the organ name as the root. For example, a benign tumor of smooth muscle cells is called a *leiomyoma* (the common name of this frequently occurring benign tumor in the uterus is *fibroid*). Confusingly, some types of cancer also use the *-oma* suffix, examples including melanoma andseminoma.

Some types of cancer are named for the size and shape of the cells under a microscope, such as giant cell carcinoma, spindle cell carcinoma, and small cell carcinoma.

PATHOLOGY

The tissue diagnosis given by the pathologist indicates the type of cell that is proliferating, its histological grade, genetic abnormalities, and other features of the tumor. Together, this information is useful to evaluate the prognosis of the patient and to choose the best treatment. Cytogenetics and immuno histochemistry are other types of testing that the pathologist may perform on the tissue specimen. These tests may provide information about the molecular changes (such as mutations, fusion genes, and numerical chromosome changes) that has happened in the cancer cells, and may thus also indicate the future behavior of the cancer (prognosis) and best treatment.

MANAGEMENT

Many management options for cancer exist with the primary ones including: surgery, chemotherapy, radiation therapy, and palliative care. Which treatments are used depends upon the type, location and grade of the cancer as well as the person's health and wishes.

SURGERY

Surgery is the primary method of treatment of most isolated solid cancers and may play a role in palliation and prolongation of survival. It is typically an important part of making the definitive diagnosis and staging the tumor as biopsies are usually required. In localized cancer surgery typically attempts to remove the entire mass along with, in certain cases, the lymph nodes in the area. For some types of cancer this is all that is needed for a good outcome.

CHEMOTHERAPY

Chemotherapy in addition to surgery has proven useful in a number of different cancer types including: breast cancer, colorectal cancer, pancreatic cancer, osteogenic sarcoma, testicular cancer, ovarian cancer, and certain lung cancers.[81] The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body.

RADIATION

Radiation therapy involves the use of ionizing radiation in an attempt to either cure or improve the symptoms of cancer. It is used in about half of all cases and the radiation can be from either internal sources in the form of brachytherapy or external sources. Radiation is typically used in addition to surgery and or chemotherapy but for certain types of cancer such as early head and neck cancer may be used alone. For painful bone metastasis it has been found to be effective in about 70% of people.

ALTERNATIVE TREATMENTS

Complementary and alternative cancer treatments are a diverse group of health care systems, practices, and products that are not part of conventional medicine and have not been shown to be effective. "Complementary medicine" refers to methods and substances used along with conventional medicine, while "alternative medicine" refers to compounds used instead of conventional medicine. Most complementary and alternative medicines for cancer have not been rigorously studied or tested. Some alternative treatments have been investigated and shown to be ineffective but still continue to be marketed and promoted.

PALLIATIVE CARE

Palliative care is an approach to symptom management that aims to reduce the physical, emotional, spiritual, and psycho-social distress experienced by people with cancer. Unlike treatment that is aimed at directly killing cancer cells, the primary goal of palliative care is to make the person feel better.

Palliative care is often confused with hospice and therefore only involved when people approach end of life. Like hospice care, palliative care attempts to help the person cope with the immediate needs and to increase the person's comfort. Unlike hospice care, palliative care does not require people to stop treatment aimed at prolonging their lives or curing the cancer. (**Astin *et al.*, 1998**)

Multiple national medical guidelines recommend early palliative care for people whose cancer has produced distressing symptoms (pain, shortness of breath, fatigue, nausea) or who need help coping with their illness. In people who have metastatic disease when first diagnosed, oncologists should consider a palliative care consult immediately. Additionally, an oncologist should consider a palliative care consult in any patient they feel has a prognosis of less than 12 months even if continuing aggressive treatment.

PROGNOSIS

Cancer has a reputation as a deadly disease. Taken as a whole, about half of people receiving treatment for invasive cancer (excluding carcinoma in situ and non-melanoma skin cancers) die from cancer or its treatment. Survival is worse in the developing world. However, the survival rates vary dramatically by type of cancer, with the range running from basically all people surviving to almost no one surviving. As of 2012, lung cancer is by far the most lethal type of cancer for men; breast cancer is the second-leading cause of cancer-related deaths for women (approximately 23 cases out of every 100,000 women), followed by colon and rectum cancer (15 out of every 100,000). Those who survive cancer are at increased risk of developing a second primary cancer at about twice the rate of those never diagnosed with cancer. The increased risk is believed to be primarily due to the same risk factors that produced the first cancer, partly due to the treatment for the first cancer, and potentially related to better compliance with screening.

Predicting either short-term or long-term survival is difficult and depends on many factors. The most important factors are the particular kind of cancer and the patient's age and overall health. People who are frail with many other health problems have lower survival rates than otherwise healthy people. A centenarian is unlikely to survive for five years even if the treatment is successful. People who report a higher quality of life tend to survive longer. People with lower quality of life may be affected by major depressive disorder and other complications from cancer treatment and/or disease progression that both impairs their quality of life and reduces their quantity of life. Additionally, patients with worse prognoses may be depressed or report a lower quality of life directly because they correctly perceive that their condition is likely to be fatal.

According to the National Cancer Institute in 2010, the most common cancers (excluding non-melanoma skin cancers) are listed below.

Cancer type	Estimated new cases	Estimated deaths
Bladder	70,530	14,680
Breast (female-male)	207,090-1,970	39,840-390
Colon and rectal (combined)	142,570	51,370
Endometrial	43,470	7,950
Kidney (renal cell)	53,581	11,997
Leukemia	43,050	21,840
Lung (including bronchus)	222,520	157,300
Melanoma	68,130	8,700
Non-Hodgkin lymphoma	65,540	20,210
Pancreatic	43,140	36,800
Prostate	217,730	32,050
Thyroid	44,670	1,690

Some other cancers include brain cancer, hodgkin's lymphoma, testicular cancer, liver cancer, uterine cancer.

The three most common cancers in men, women and children are as follows:

- **Men**: Prostate, lung, and colorectal.
- **Women**: Breast, colorectal, and lung.
- **Children**: Leukemia, brain tumours, and lymphoma. **(Jermal *et al.*, 2011)**

PREVENTION

Cancer prevention is defined as active measures to decrease the risk of cancer.[43] The vast majority of cancer risk factors are due to environmental (including lifestyle) factors, and many of these factors are controllable. Thus, cancer is largely considered a preventable disease. Greater than 30% of cancer is considered preventable by avoiding risk factors including: tobacco, overweight / obesity, an insufficient diet, physical inactivity, alcohol, sexually transmitted infections, and air pollution. Not all environmental causes can be prevented completely such as naturally occurring background radiation.

DIETARY

While many dietary recommendations have been proposed to reduce the risk of cancer, few have significant supporting scientific evidence. The primary dietary factors that increase risk are obesity and alcohol consumption; with a diet low in fruits and vegetables and high in red meat being implicated but not confirmed. Consumption of coffee is associated with a reduced risk of liver cancer. Studies have linked consumption of red or processed meat to an increased risk of breast cancer, colon cancer, and pancreatic cancer, a phenomenon which could be due to the presence of carcinogens in foods cooked at high temperatures. Thus dietary recommendation for cancer prevention typically include: "mainly vegetables, fruit, whole grain and fish and a reduced intake of red meat, animal fat and refined sugar."

MEDICATION

The concept that medications can be used to prevent cancer is attractive, and evidence supports their use in a few defined circumstances. In the general population NSAIDs reduce the risk of colorectal cancer however due to the cardiovascular and gastrointestinal side effects they cause overall harm when used for prevention. Aspirin has been found to reduce the risk of death from cancer by about 7%.COX-2 inhibitor may decrease the rate of polyp formation in people with familial adenomatous polyposis however are associated with the same adverse effects as NSAIDs. Daily use of tamoxifen or raloxifene has been demonstrated to

reduce the risk of developing breast cancer in high-risk women. The benefit verses harm for 5-alpha-reductase inhibitor such as finasteride is not clear.

Vitamins have not been found to be effective at preventing cancer, although low blood levels of vitamin D are correlated with increased cancer risk. Whether this relationship is causal and vitamin D supplementation is protective is not determined. Beta-carotene supplementation has been found to increase lung cancer rates in those who are high risk. Folic acid supplementation has not been found effective in preventing colon cancer and may increase colon polyps.[63]

VACCINATION

Vaccines have been developed that prevent some infection by some viruses. Human papillomavirus vaccine (Gardasil and Cervarix) decreases the risk of developing cervical cancer. The hepatitis B vaccine prevents infection with hepatitis B virus and thus decreases the risk of liver cancer. **(Thum et. al. 2007)**

HOW CELLS DIE: APOPTOSIS AND OTHER

Cell death is an essential part of normal development and continues into adulthood. The human body, for instance, is composed of approximately 10^{14} cells. Every day billions of cells die an altruistic death in order to secure the functionality of the whole organism. Thus, we remain the same size only because cell division exactly balances cell death.

During development, cell death helps sculpt organs or separate fingers and toes. It also eliminates structures that once served a function but are no longer needed, such as the tail of a tadpole during amphibian metamorphosis. Most of the neurons die during development before having any chance to function in the nervous system. Cell death also eliminates most newly formed lymphocytes, especially those that are useless or dangerous, by targeting self-antigens. Neutrophils, for instance, are produced continuously in the bone marrow, but the vast majority die within a few days. This apparently futile cycle of cell proliferation and cell death serves to maintain a supply of cells that can be readily mobilized when needed .

As cell death is intimately linked to tissue homeostasis, its disruption has, not surprisingly, been implicated in numerous pathological conditions. A reasonable estimate is that either too little or too much cell death contributes to approximately half of all medical illnesses, for many of which no adequate therapy exists. Abnormalities in cell death regulation can be a significant component of diseases such as cancer, autoimmune syndromes, AIDS, ischemia, liver diseases and neurodegenerative disorders including Parkinson's and Alzheimer's disease . Consequently, considerable interest has emerged in devising therapeutic strategies aimed at modulating cellular life-and-death decisions.

TERMINOLOGY OF CELL DEATH

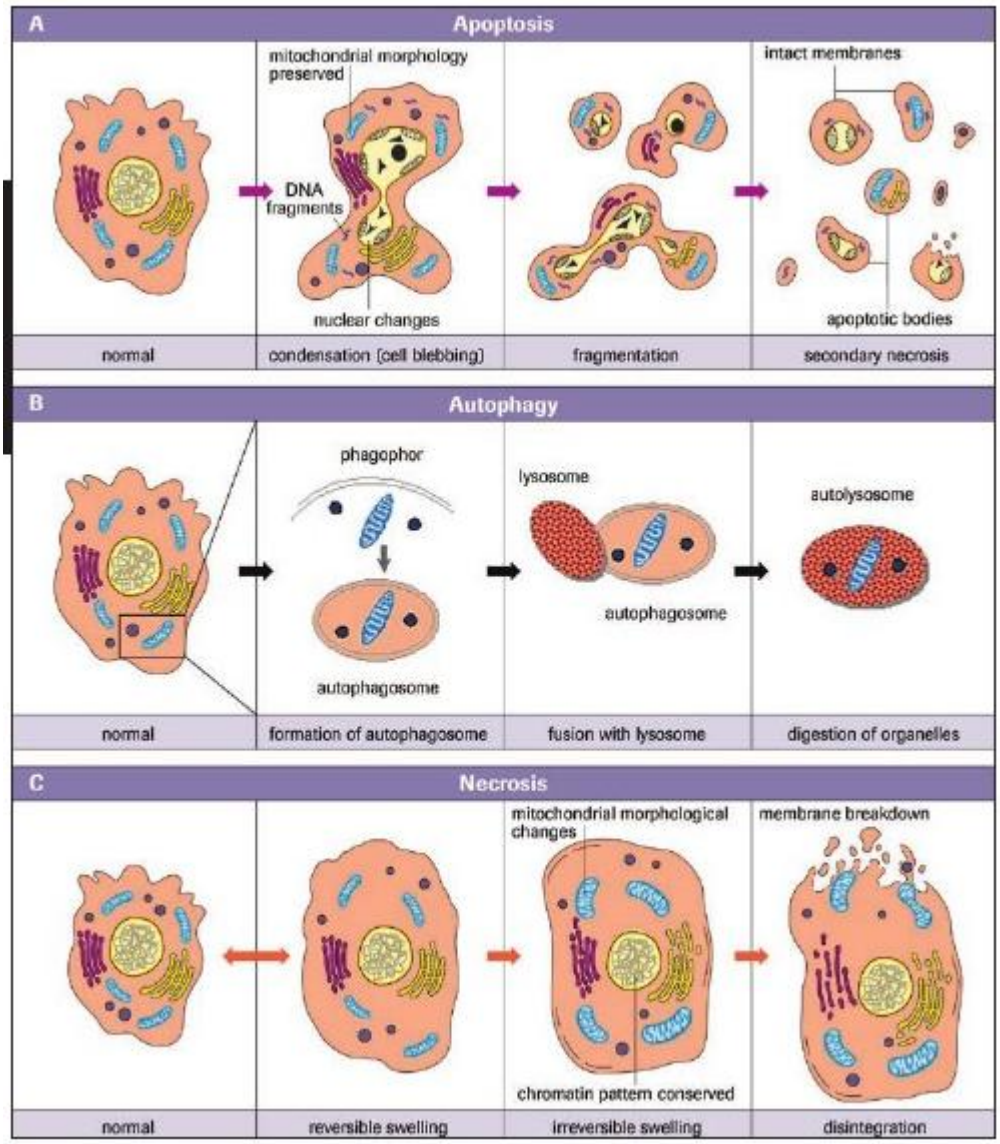
Apoptosis, also termed type I cell death, is defined by characteristic changes in the nuclear morphology, including chromatin condensation and fragmentation, overall cell shrinkage, blebbing of the plasma membrane and formation of apoptotic bodies that contain nuclear or cytoplasmic material

Autophagic cell death, also known as type II cell death, is characterized by a massive accumulation of double-membrane containing vacuoles known as autophagosomes, which subsequently fuse with lysosome vacuoles.

Type III cell death, better known as **necrosis**, is often defined in a negative manner as death lacking the characteristics of the type I and type II processes.

Distinction of different cell death forms is not only relevant for semantic reasons, but can also have important clinical implications when considering the potential therapeutic targeting of cell death processes. Nevertheless, in some conditions these distinct cell modes may only be the extremes, and there are numerous examples in which cell death demonstrates a continuum of intermediate features, for instance of both apoptosis and necrosis..

Furthermore, in some cases it is the cell type or the nature and duration of cellular injury that determine whether cells die by apoptosis, necrosis or other default mechanisms. At low doses, a variety of harmful stimuli such as radiation, hypoxia and anticancer drugs can induce apoptosis, but the same stimuli can result in necrosis at higher doses. Therefore, in many situations cell death may not occur as a clear-cut and paradigmatic form of cell death.



APOPTOSIS

Apoptosis occurs in a well-choreographed sequence of morphological events. This process usually starts with the blebbing of the plasma membrane, which breaks up into membrane-enclosed particles, termed apoptotic bodies, containing intact organelles as well as portions of the nucleus. In fact the word 'apoptosis' comes from the ancient Greek, meaning 'falling off ' (of petals from a flower) and refers to the morphological feature of the formation of apoptotic bodies . These apoptotic bodies are rapidly recognized, ingested and eaten by professional phagocytes or neighboring cells. Under physiological conditions certain modifications occur in the

plasma membrane which function as ‘eat-me’ signals and enable the apoptotic bodies to be recognized by phagocytic cells. Since the apoptotic bodies are surrounded by an intact plasma membrane, apoptosis usually occurs without any leakage of cellular contents and therefore without provoking an inflammatory response. Moreover, the engulfment of apoptotic cells by macrophages triggers the production of anti-inflammatory cytokines.

Because apoptotic cells are eaten and digested so quickly, there are usually few dead cells to be seen in tissue sections, even when large numbers of cells have died. This probably explains why apoptosis was neglected by pathologists for a long time. Looking inside the cell, one of the most noticeable features of apoptosis is the condensation of the nucleus and its fragmentation into smaller pieces, a highly distinctive event that is not seen in other forms of cell death. Another defining feature is the extensive hydrolysis of nuclear DNA into internucleosomal fragments .

Apoptosis is the major cell death pathway for removing unwanted and harmful cells in a clean or silent manner during embryonic development, tissue homeostasis and immune regulation. In addition, most anti-cancer therapies rely on the activation of apoptotic pathways. As the alterations of apoptosis are stereotypical and similar in all cell types irrespective of the death stimulus, the biochemical mechanisms underlying these changes also follow a similar built-in program. In nematodes, insects and human cells, most, if not all, morphological alterations of apoptosis are mediated by the activation of an evolutionarily conserved and unique class of intracellular proteases known as caspases .

AUTOPHAGY

Like apoptosis, autophagy is a highly conserved and genetically controlled process involving a cascade of molecular events. Autophagy (meaning self-eating) is classically activated in response to nutrient starvation, but is also observed during development, differentiation and various forms of environmental stress. In addition, defective autophagy underlies a number of pathological conditions, including myopa-thies, neurodegenerative diseases, liver diseases, and some forms of cancer .

Autophagy is a major catabolic mechanism by which long-lived proteins and organelle components are directed to lysosomes and recycled in order to maintain energy and protein synthesis. It is characterized by the appearance of numerous cytosolic vacuole-like structures known as autophagosomes, which are formed by the assembly of double-layered, membrane-bound structures of still largely undefined origin. The autophago-somes encapsulate cytosolic materials and subsequently fuse with lysosomes, which causes the autophagosomal contents to degrade .

Although the role of the autophagic process in protein and organelle degradation, and inprotection during nutrient starvation is readily accepted, its function in programmed cell death is controversial . This is partly because the term 'autophagic cell death' hasbeen applied to two distinct observations: cell death associated with autophagy and cell death through autophagy.

Under normal physiological conditions autophagy occurs at low basal levels, contribut-ing to the turnover of cytoplasmic components and promoting cell adaptation and survival during stress, e.g. starvation. Excess autophagy, on the other hand, leads to autophagic cell death. Interestingly, in cells deficient for apoptosis, apoptotic signals can trigger massive autophagy and cell death, which is prevented by the inhibition of autophagosome formation. Thus, a complex relationship also exists between autophagy and apoptosis, in that autophagy can both promote and inhibit apoptotic cell death. Interestingly, some of the regulators of apoptosis also interfere with autophagic processes . At present, it would seem that, while the predominant function of autophagy is to promote cell survival, extended autophagy will result in autophagic cell death. Clearly, more work is needed to elucidate the role of autophagy as a cell death mechanism and the complex crosstalk with other cell death pathways.

NECROSIS

Necrotic cell death is often defined negatively as a form of cell death that lacks signs of apoptosis or autophagy. Necrotic cells typically show cytoplasmic swelling and vacuolation, rupture of the plasma membrane, dilation of organelles (mitochondria, endoplasmic reticulum and Golgi apparatus), as well as moderate chromatin condensation. When cells swell and burst they spill their contents over their neighbors and elicit a damaging inflammatory response.

Necrosis is usually considered to be an uncontrolled and accidental cell death which, unlike apoptosis, is not energy-dependent. Biochemically, most prominent features include massive energy depletion, the formation of reactive oxygen species and the activation of non-apoptotic proteases. All these events result in a loss of function of homeostatic ion pumps and damage to membrane lipids with cell membrane swelling and rupture

Furthermore, during necrosis a substantial rise in intracellular calcium is observed. The elevated calcium levels in the cytosol trigger mitochondrial calcium overload, leading to depolarization of the inner mitochondrial membrane and a shut-down of ATP production . While depletion of ATP impedes the function of membrane channels, increased calcium activates calcium-dependent proteases, e.g. calpains. Calcium fluxes, ATP depletion and oxidative stress involve complex and interactive feedback loops that self-amplify and potentiate each other, leading to exaggerated cell death. Such processes are most relevant under conditions of excessive trauma and ischemia-reperfusion. Necrosis, however, can also be observed in response to death receptor activation or chemotherapy, conditions that were originally believed to mediate cell death exclusively via apoptosis.

In addition, the inhibition of specific proteins involved in regulating apoptosis or autophagy can switch the type of cell death to necrosis. Finally, secondary or post-apop-totic necrosis occurs when massive apoptosis overwhelms the scavenging activity of phagocytes, thereby resulting in leakage of the cell contents with induction of inflamma-tory responses.

There is increasing evidence that necrosis is more tightly regulated than previously thought and underlies a genetic control that might be relevant in multiple physiological and pathological scenarios . Necrosis might therefore serve as a backup cell death mechanism when apoptosis or autophagic cell death fails.

TERMINOLOGY OF CELL PROLIFERATION AND VIABILITY

Rapid and accurate assessment of viable cell number and cell proliferation is an impor- tant requirement in many experimental situations involving in vitro and in vivo studies. Examples of where determination of cell number is useful include the analysis of growth factor activity, serum batch testing, drug screening, and the determination of the cytostatic potential of anti-cancer compounds in toxicology testing. In such toxicological studies, in vitro testing techniques are very useful to evaluate the cytotoxic, mutagenic, and carcinogenic effects of chemical compounds on human cells.

Usually, one of two parameters is used to measure the health of cells: cell viability or cell proliferation. In almost all cases, these parameters are measured by assaying for “vital functions” that are characteristic of healthy cells.

CELL VIABILITY

Cell viability can be defined as the number of healthy cells in a sample. Whether the cells are actively dividing or are quiescent is not distinguished. Cell viability assays are often useful when non-dividing cells (such as primary cells) are isolated and maintained in culture to determine optimal culture conditions for cell populations.

The most straightforward method for determining viable cell number is a direct counting of the cells in a hemocytometer. Sometimes viable cells are scored based on morphology alone; however, it is more helpful to stain the cells with a dye such as trypan blue. In this case, viability is measured by the ability of cells with uncompromised membrane integrity to exclude the dye.

Alternatively, metabolic activity can be assayed as an indication of cell viability. Usually metabolic activity is measured in populations of cells by incubating the cells with a tetrazolium salt (MTT, XTT, WST-1) that is cleaved into a colored formazan product by metabolic activity.

CELL PROLIFERATION

Cell proliferation is the measurement of the number of cells that are dividing in a culture. One way of measuring this parameter is by performing clonogenic assays. In these assays, a defined number of cells are plated onto the appropriate matrix and the number of colonies that are formed after a period of growth are enumerated. Drawbacks to this type of technique are that it is tedious and it is not practical for large numbers of samples. In addition, if cells divide only a few times and then become quiescent, colonies may be too small to be counted and the number of dividing cells may be underestimated. Alternatively, growth curves could be established, which is also time-consuming and laborious.

Another way to analyze cell proliferation is the measurement of DNA synthesis as a marker for proliferation. In these assays, labeled DNA precursors (3H-thymidine or bromodeoxyuridine) are added to cells and their incorporation into DNA is quantified after incubation. The amount of labeled precursor incorporated into DNA is quantified either by measuring the total amount of labeled DNA in a population, or by detecting the labeled nuclei microscopically. Incorporation of the labeled precursor into DNA is directly proportional to the amount of cell division occurring in the culture.

Cell proliferation can also be measured using more indirect parameters. In these techniques, molecules that regulate the cell cycle are measured either by their activity (e.g., CDK kinase assays) or by quantifying their amounts (e.g., Western blots, ELISA, or immunohistochemistry).

WHY THE PRESENT COMPOUNDS ARE TAKEN FOR ANTITUMOR ACTIVITY?

Silibinin, a flavonone which is the class of flavonoid, has shown the prominent activity on the cancerous cell lines in previous studies. Silibinin has shown the cell growth inhibition in cell culture by alteration of cell cycle progression and inhibition of mitogenic and cell survival signaling. Silibinin also found to inhibit the secretion of proangiogenic factor like Epidermal growth factor, insulin growth factor, nuclear factor kappa β from tumor cell.

Genistein, a phytoestrogen which comes under the isoflavonone class of flavonoids, has shown the inhibition of tyrosin kinase, and mostly the epidermal derived growth factor. Which in turn destroys the cell signaling cascade and proliferation of cells. Genistein has also shown the cytoprotective activity by reducing the lipid peroxidaion.

This data supports the study of the silibinin and genistein to be tested together for its cytotoxic activity in combined as it has never been performed on breast and colon cell lines. The cytoproctive activity is assessed on the basis of trial and error basis, because the compound silibinin hasn't shown any kind of cytoproctive activity until now.

So these compounds are choosan for the cytotoxic and cytoprotective activity in single and in combination treatments.

FLAVONOIDS

Flavonoids (or **bioflavonoids**) (from the Latin word *flavus* meaning yellow), also collectively known as **Vitamin P** and **citrin**, are a class of plant secondary metabolites or yellow pigments having a structure similar to that of flavones. According to the IUPAC nomenclature they can be classified into:

- *flavonoids*, derived from 2-phenylchromen-4-one (2-phenyl-1, 4-benzopyrone) structure (examples: quercetin, rutin).
- *isoflavonoids*, derived from 3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone) structure
- *neoflavonoids*, derived from 4-phenylcoumarine (4-phenyl-1,2-benzopyrone) structure. (Flavanoids IUPAC compendium of chemical technology)

The three flavanoids classes above are all ketone-containing compounds, and as such, are flavanoids and flavonol.

Flavonoids are the most important plant pigments for flower coloration producing yellow or red/blue pigmentation in petals designed to attract pollinator animals.

Flavonoids secreted by the root of their host plant help *Rhizobia* in the infection stage of their symbiotic relationship with legumes like peas, beans, clover, and soy.

In addition, some flavanoids have inhibitory activity against organisms that cause plant disease e.g. *Fusarium oxysporum*.

1). SOURCES

Good sources of flavanoids include all citrus fruits, berries, ginkgo biloba, onions (particularly red onion), parsley, pulses, tea (especially white and green tea), red wine, sea buckthorn, and dark chocolate (with a cocoa content of seventy percent or greater).

Flavonoids (specifically flav**a**noids such as the catechins) are "the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants". Flavonols, the original bioflavonoids such as quercetin, are also found ubiquitously, but in lesser quantities. **(Brown, 1980**), **(Justesen *et al*., 2001)**

2). EFFECTS ON HUMAN HEALTH

Flavonoids might induce mechanisms that affect cancer cells and inhibit tumor invasion. In preliminary studies, UCLA cancer researchers proposed that smokers who ate foods containing certain flavanoids, such as catechins found in strawberries and green and black teas, kaempferol from brussel sprouts and apples, and quercetin from beans, onions and apples, may have reduced risk of obtaining lung cancer.

The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Preliminary research indicates that flavonoids may modify allergens, viruses, and carcinogens, and so may be biological "response modifiers". In vitro studies show that flavonoids also have anti-allergic, anti-inflammatory, anti-microbial, anti-cancer, and anti-diarrheal activities.

3). ANTIOXIDANT ACTIVITY *INVITRO*

Flavonoids (both flav**o**nols and flav**a**nols) are most commonly known for their antioxidant activity. Additionally, at high experimental concentrations that would not exist in vivo, the antioxidant abilities of flavanoids in vitro are stronger than those of vitamin C & E.

4). WHAT DO THEY DO?

As antioxidants, some flavanoids such as Quercetin protect LDL cholesterol from oxidative damage.

The ability to limit inflammation gives the flavanoids their disease fighting potential. They actively prevent the release of histamine in the body - this is the agent responsible for most allergy symptoms like congestion.

Flavonoids also actively scavenge free radicals they tend to boost immune system functioning and strengthen the blood vessels, thereby increasing the blood flow.

Flavonoids exhibit potent anticancer effects, although the exact target of such inhibition has not been definitely established, given the recent evidence for a prominent role for reactive oxygen species (ROS) in carcinogenesis. It is tempting to speculate that flavanoids inhibit carcinogenesis due to ROS scavenging. However there have been a number of reports that directly contradict the potential role of flavanoids as antioxidants/anticancer agents.

Flavonoid and related compound are effective in scavenging DPPH radical, hydroxyl radical and in metal-chelating capacity. **(Irfan khan 2011)**

SYNTHETIC DRUGS

Chemotherapy is the treatment of an ailment by chemicals especially by killing micro-organisms and other malfunctions of the body.

The majority of chemotherapeutic drugs can be divided in to alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents. All of these drugs affect cell division or DNA synthesis and function in some way.

Some newer agents do not directly interfere with DNA. These include monoclonal antibodies and the new tyrosine kinase inhibitors e.g. *imatinib mesylate* (*Gleevec* or *Glivec*), which directly targets a molecular abnormality in certain types of cancer (chronic myelogenous leukemia, gastrointestinal stromal tumours). These are examples of targeted therapies. **(Tripathi, 2008)**

In addition, some drugs that modulate tumor cell behaviour without directly attacking those cells may be used. Hormone treatments fall into this category.

The different types of anti cancer agents used in chemotherapy are as follows

TYPES	**EXAMPLES**
Alkylating agents	Cisplatin, carboplatin and oxaliplatin
Anti-metabolites	Purines - azathioprine, mercaptopurine Pyramidines - 5-fluro uracil
Plant alkaloids	Vincristine, vinblastine, vindesine, vinorelbine
Podophyllotoxin	Etopside, teniposide
Taxanes	Docetaxel

Topisomerase inhibitors	Type 1 – irinotecan, topotecan Type 2- amasacrine, etopside
Cytotoxic antibiotics	Actinomycin, anthracyclines, belomycin
Others	Hydroxyurea, procarbazine, carboplatin,cisplatin
Drugs atering hormonal milieu	Prednisolone, tamoxifen, hydroxyl progesterone, fosfestrol, letrozole etc

DIFFERENT METHODS FOR SCREENING OF NEW ANTICANCER MOLECULES

The main aims of screening methods are

a. To test the ability of a compound to kill cancerous cells.
b. To discriminate between replicating and non-replicating cells.
c. To find the potency of the drug
d. Effectiveness of drug and specific site of action

The different methods of screening are

1. In-vitro methods
2. In-vivo methods
3. Cell line methods

1) INVITRO METHODS

In-vitro testing is preferred to in-vivo testing for testing the potential of chemotherapeutic agents. In-vitro cultures can be cultivated under a controlled environment (pH, temperature, humidity, oxygen/carbon dioxide balance etc) resulting in homogenous batches of cells and reducing the mistakes.

Different types of in-vitro methods include

1. Tetrazolium salt assay(MTT)
2. Sulphorhodamine B assay
3. ^{3}H thymidine uptake
4. Dye exclusion tests
5. Clonogenic assay
6. Cell counting assay

These methods used to find different type of properties in culture cells and thus used to detect the therapeutic effect of the drug and potency. These tests are frequently used for screening of anti-cancer drugs:

S.NO	ASSAY	PROPERTIES
1	Tetrazolium salt assay(MTT)	Enzymatic properties
2.	Sulphorhodamine B assay	Protein content and synthesis
3.	^{3}H thymidine uptake	DNA content and synthesis
4.	Dye exclusion assay	Membrane integrity of cell
5.	Clonogenic assay	Clonogenic properties
6.	Cell counting assay	Cell divison

2) INVIVO METHODS

The in-vivo testing is performed on animals to test the efficacy of the drug on animals and to check the different parameters and compare them with the standard.

1. DMBA induced mouse skin papillomas, rat mammary gland carcinogenesis, oral cancer in hamster.
2. N-methyl, N- nitrosurea(NMU) induced rat mammary gland carcinogenesis.
3. NMU induced tracheal squamous cell carcinoma in hamster.
4. N, N diethyl nitrosamine (DEN) induced lung asenocarcinoma in hamster.
5. 1, 2 Dimethyl hydralazine (DMH) inducer colorectal adenocarcinoma in rat and mouse.

6. Azoxymethane (AOM) induced aberrant crypt foci in rats.
7. 1- Butyl-n-(4 hydroxy butyl)- nitrosamine induced bladder carcinoma in mouse.
8. 2- Methylcholanthrene induced fibrosarcoma tumor in mouse.
9. 3-Methlcholanthrene induced skin tumours in mouse.
10. Benzopyrene induced fore stomach tumours in mouse.

In all these in-vivo methods the cancer is induced by some chemicals and then the drug given by different routes and the efficacy measured and compared with that of the standard drugs and thus the activity of the drug found out statistically.

3) CELL LINE METHODS

In this method of studies the cancer cells are directly induced into the animal and propagated to induce cancer in the animals. The specified number of cells inoculated in to a sensitive mouse strains and tumor developed rapidly as compared to chemical carcinogen. It is much faster and less time consuming model than other methods.

Different types of cell line techniques are

1. Hollow fiber technique.
2. Use of kenografts.
3. Nude mouse models.
4. New born rat model.
5. Transgenic mouse model.
6. DAL induced mouse model. **(Gupta, 2008)**

a) DAL (DALTON'S ASCITES LYMPHOMA) MOUSE MODEL

Ascites is excess fluid in the space between the tissues lining the abdomen and abdominal organs (the peritoneal cavity). (**Ascites medline plus**, A service of US national library of Medicine NIH national institute of health)

Lymphomas are cancers that begin in the cells of the immune system

- It is a tumor cell line originally grown from a tumour of the thymus.
- It is propagated by growing as ascites tumour in mice.
- We can induce both ascites tumour and solid tumours using DAL cells.
- It is easy to maintain in vivo.
- It is not an immunogenic.

The solid tumours were obtained by injecting the DAL cell lines subcutaneously in to the mouse hind paw which gets increased in the mass and becomes solid. This cannot be propagated like the ascites lymphoma.

b) MAINTENANCE OF CELL LINES:

Dalton's lymphoma ascites tumour cell lines (DLA), originally obtained from Amala Cancer Institute, Thrissur, Kerala and was propagated as transplantable tumours in the peritoneal cavity of the mice were used for the study.

The tumour cell lines were maintained by serial peritoneal cavity I.P transplantation in mice.

The fully grown tumour cell lines were aspirated from mouse peritoneal cavity and mixed with PBS solution and mixed with tryphan blue solution. Taken that suspended solution and counted the number of cells present in one ml by using tryphan blue exclusion method and adjust the cell count to $1\times10^{6.}$ Then PBS solution was used and the cells were mixed well and were injected intraperitonially in to a new healthy mouse and thus the cancer got developed with in 10-15days of time.

LITERATURE REVIEW

- Yun-Hee Shon et al., 2006 reported about the "Effective Chemopreventive Activity of Genistein against Human Breast Cancer Cells"

  "The author repored that on breast cancer cell lines Genistein inhibited cell proliferation in estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) human breast carcinoma cell lines. Cytochrome P450 (CYP) 1A1-mediated ethoxyresorufin O-deethylase (EROD) activity was inhibited by genistein in a concentration-dependent manner. Genistein significantly inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cyclooxy-enase-2 activity and protein expression at the concentrations of 10 ($p < 0.05$), 25 ($p < 0.05$) and 50 mM ($p < 0.01$). Inaddition, ornithine decarboxylase (ODC) activity was reduced to 53.8 % of the control after 6 h treatment with 50 mM genistein in MCF-7 breast cancer cells. These results suggest that genistein could be of therapeutic value in preventing human breast cancer."

- Rana P. Singh et al., 2004 reported about the "Prostate Cancer Prevention by Silibinin"

  "The author reported that for PCA prevention, growth control and/or treatment could be inhibition of epigenetic molecular events involved in PCA growth, progression and angiogenesis. In this regard, silibinin/silymarin (silibinin is the major active compound in silymarin) has shown promising efficacy. Our extensive studies with silibinin/silymarin and PCA cells have shown the pleiotropic anticancer effects leading to cell growth inhibition in culture and nude mice. The underlying mechanisms of silibinin/silymarin efficacy against PCA involve alteration in cell cycle progression, and inhibition of mitogenic and cell survival signaling, such as epidermal growth factor receptor, insulin-like growth factor receptor type I and nuclear factor kappa B signaling. Silibinin also synergizes the therapeutic effects of doxorubicin in PCA cells, making it a strong candidate for combination chemotherapy. Silibinin/ silymarin also inhibits the secretion of proangiogenic

factors from tumor cells, and causes growth inhibition and apoptotic death of endothelial cells accompanied by disruption of capillary tube formation on Matrigel. More importantly, silibinin inhibits the growth of in vivo advanced human prostate tumor xenograft in nude mice."

- Leyon Varghese et al., 2005 reported about the "Silibinin Efficacy against Human Hepatocellular Carcinoma"

  "The author reported that Silibinin strongly inhibited growth of both HepG2 and Hep3B cells with a relatively stronger cytotoxicity in Hep3B cells, which was associated with apoptosis induction. Silibinin also caused G1 arrest in HepG2 and both G1 and G2-M arrests in Hep3B cells. Mechanistic studies revealed that silibinin induces Kip1/p27 but decreases cyclin D1, cyclin D3, cyclin E, cyclin-dependent kinase (CDK)-2, and CDK4 levels in both cell lines. In Hep3B cells, silibinin also reduced the proteinlevels of G2-Mregulators. Furthermore, silibinin strongly inhibited CDK2, CDK4, and CDC2 kinase activity in these HCC cells. Conclusion: Together, these results for the first time identify the biological efficacy of silibinin against HCC cells, suggesting the importance of conducting further investigations in preclinical HCC models, especially on in vivo efficacy, to support the clinical usefulness of silibinin against hepatocellular carcinoma in addition to its known clinical efficacy as an antihepatotoxic agent."

- Noël J.-M. Raynal et al., 2007 reported about the "Antileukemic Activity of Genistein, a Major Isoflavone Present in Soy Products"

  " The author reported the in vitro and in vivo antileukemic activity of genistein , a major isoflavone present in soy. Observed that it produced a dose- and time-dependent antineoplastic activity against myeloid and lymphoid leukemic cell lines. In addition, genistein treatment of the leukemic cells reactivated tumor suppressor genes that were silenced by aberrant DNA methylation. A genistein-enriched diet produced a moderate, but significant, antileukemic effect in mice. The limited extent of this in vivo response may have been due to the rapid metabolic inactivation of genistein in mice. Due to the longer half-life of genistein in humans, a soy-enriched diet has the potential

to produce plasma levels of this isoflavone in the range of the concentrations used in vitro that produced an antileukemic activity.".

- Rana P. Singh et al., 2001 reported about the "Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin 3-gallate on epigenetic events in human prostate carcinoma DU145 cells."

  "The author reported that Treatment of cells with silymarin, genistein or EGCG at 100–200 μM resulted in a complete inhibition of TGFα-caused activation of erbB1 followed by a moderate to strong inhibition (10–90%) of Shc activation without an alteration in their protein levels. Silymarin and genistein, but not EGCG, also inhibited (10% to complete) ERK1/2 activation suggesting that these agents impair erbB1-Shc-ERK1/2 signaling in DU145 cells. In other studies, silymarin, genistein or EGCG caused a strong induction of Cip1/p21 (up to 2.4-fold) and Kip1/p27 (up to 150-fold), and a strong decrease in CDK4 (40–90%) but had moderate effect on CDK2, and cyclins D1 and E. An enhanced level of CDKIs also led to an increase in their binding to CDK4 and CDK2. Treatment of cells with silymarin, genistein or EGCG also resulted in 50–80% cell growth inhibition at lower doses, and complete inhibition at higher doses. In contrast to silymarin, higher doses of genistein showed cytotoxic effect causing 30–40% cell death. A more profound cytotoxic effect was observed with EGCG accounting for 50% cell death at lower doses and complete loss of viability at higher doses."

- Ian R. Record et al., 1995 reported about the "The antioxidant activity of genistein in vitro. "

  "The author reported that genestein proved to be effective against UVA and UVB or peroxyl radical-induced lipid peroxidation in liposomes. Genistein was however ineffective in preventing conjugated diene formation in linoleic acid micelles. Other peroxidative systems involving hydrogen peroxide, such as metmyoglobin peroxidase activity and Fel ascorbate/hydrogen peroxide oxidation of liposomes, were inhibited by genistein. As measured by catechol decolorization, genistein did not appear to chelate iron. Genistein removed hydrogen peroxide efficiently when phenol red was coupled with peroxidase;

however, when o-dianisidine was used as the color reagent there was no apparent loss of hydrogen peroxide, possible due to oxidation of the dye by the product of genistein and hydrogen peroxide. This study provides further evidence that genistein is an effective scavenger of hydrogen peroxide but is less effective against other peroxidative systems."

- Aslamuzzaman kazi et al., 2003 reported about the "Inhibition of the proteasome activity, a novel mechanism associated with the tumor cell apoptosis-inducing ability of genistein."

  "The author reported that genistein inhibits the proteasomal chymotrypsin like activity *in vitro* and *in vivo*. Computational docking studies suggest that the interaction of genistein with the proteasomal β5 subunit is responsible for inhibition of the chymotrypsin-like activity. Inhibition of the proteasome by genistein in prostate cancer LNCaP and breast cancer MCF-7 cells is associated with accumulation of ubiquitinated proteins and three known proteasome target proteins, the cyclin-dependent kinase inhibitor $p27^{Kip1}$, inhibitor of nuclear factor-κB (IκB-α), and the pro-apoptotic protein Bax. Genistein-mediated proteasome inhibition was accompanied by induction of apoptosis in these solid tumor cells. Finally, genistein induced proteasome inhibition and apoptosis selectively in simian virus 40-transformed human fibroblasts, but not in their parental normal counterpart. Our results suggest that the proteasome is a potential target of genistein in human tumor cells and that inhibition of the proteasome activity by genistein might contribute to its cancer-preventive properties."

- M.A. Louis Jeune et al., 2005 reported about the "Anticancer Activty of promigranets extracts and genistein on human breast cancer cell lines."

  "The author reported that pomegranate extracts and genistein had significant (dose- and time-dependent) cytotoxic and growth inhibition effects on MCF-7 cancer cells. Both growth inhibition and cytotoxicity were significantly higher ($P < .01$) in the combination treatments than in the single treatments with either agent. The data revealed that both drugs in single and in combination treatments induced apoptosis in MCF-7 cells. Apoptotic induction in the

combination treatments was significantly higher ($P < .01$) than in single treatments. Both pomegranate extracts and genistein inhibit the growth of MCF-7 breast cancer cells through induction of apoptosis, with combination treatment being more efficacious than single treatments."

- Li, Yiwei MD et al., 2004 reported about "Apoptosis-Inducing Effect of Chemotherapeutic Agents Is Potentiated by Soy Isoflavone Genistein, a Natural Inhibitor of NF-[kappa]B in BxPC-3 Pancreatic Cancer Cell Line."

  "The author reported that Genistein has been shown to inhibit the growth of various cancer cells in vitro and in vivo without toxicity to normal cells. The antitumor effects of genistein could be in part due to inactivation of NF-κB activity. In contrast, chemotherapeutic agents inadvertently induce NF-κB activity, which may lead to chemoresistance. In this study, we investigated whether the inactivation of NF-κB by genistein would enhance the efficacy of chemotherapeutic agents. The combination of 30 μmol/L genistein with 1 nmol/L docetaxel or 100 nmol/L cisplatin elicited significantly greater inhibition of cell growth compared with either agent alone. The combination treatment induced more apoptosis in BxPC-3 cells compared with single agents. Moreover, the NF-κB activity was significantly increased within 2 hours of docetaxel or cisplatin treatment, and the NF-κB-inducing activity of these agents was completely abrogated in cells pretreated with genistein. These results clearly suggest that genistein pretreatment, which inactivates NF-κB activity, together with other cellular effects of genistein, may contribute to increased cell growth inhibition and apoptosis inducing effects of nontoxic doses of docetaxel and cisplatin, which could be a novel strategy for the treatment of pancreatic cancer."

- Fatih M. Uckun et al., 1998 reported about the "In Vivo Toxicity, Pharmacokinetics, and Anticancer Activityof Genistein Linked to Recombinant Human Epidermal Growth Factor"

  "The author reported that Epidermal growth factor receptor (EGFR)-associated protein tyrosine kinase (PTK) complexes have vital anti- apoptotic

functions in human breast cancer cells. We have shown previously that targeting the naturally occurring PTK inhibitor genistein to the EGFR-associated PTK corn- plexes using the EGF-Genistein (Gen) conjugate triggers rapid apoptotic cell death in human breast cancer cells and abrogates their in vitro clonogenic growth. In the present study, we examined the in vivo toxicity profile, pharmaco- Idnetics, and anticancer activity of EGF-Gen. No toxicities were observed in mice treated with EGF-Gen at dose levels as high as 40 mgfkg administered i.p. as a single dose or 140 mg/kg administered i.p. over 28 consecutive days. EGF-Gen significantly improved tumor-free survival in a severe combined immune deficiency (SCm) mouse xenograft model of human breast cancer"

- Anil K. Tyagi et al., 2002 reported about the " Silibinin Strongly Synergizes Human Prostate Carcinoma DU145 Cells to Doxorubicin-induced Growth Inhibition, G_2-M Arrest, and Apoptosis"

"The author reported that Silibinin strongly synergized the growth-inhibitory effect of doxorubicin in prostate carcinoma DU145 cells ,which was associated with a strong G2-M arrest in cell cycle progression, showing 88%cells in G2-Mphase by this combination compared with 19 and 41% of cells in silibinin and doxorubicin treatment alone, respectively. The underlying mechanism of G2-M arrest showed a strong inhibitory effect of combination on cdc25C, cdc2/p34, and cyclin B1 protein expression and cdc2/p34 kinase activity. More importantly, this combination caused 41%apoptotic cell death compared with 15%by either agent alone. Silibinin and doxorubicin alone as well as in combination were also effective in inhibiting the growth of androgen-dependent prostate carcinoma LNCaP cells."

AIM AND OBJECTIVE

The major aim of this study is to evaluate the antitumor activity of the two flavonoids 1.Silibinin 2.genestein in combined on breast cancer cell lines and colon cancer cell lines. The silibinin and genestein has shown the antitumor activity on prostate cancer in combined.

Silibinin and Genestein has shown the cell growth inhibition in culture by alteration of cell cycle progression and inhibition of mitogenic and cell survival signaling. Both the compounds have shown the proangiogenic factor from tumor cell.

Cancer is one of the dangerous, lethal and a major challenging disease to medicinal system to produce more potent and the site specific anti-cancer drugs. Vast number investigations are going on to find out the potent and safer new anti-cancer drugs.

Flavanoids have a capacity to elevate the antioxidant levels and prevent the free radical reactions and also reported that flavanoids have an antitumor activity. Flavanoids are also called as free radical scavengers because of their activity on the free radicals and ROS. Hence in the present study *silibinin and genestein* which are novel flavanoids was used for evaluating the antitumor activity due to its free radical scavenging activity since many studies done previously on flavanoid antioxidant and antitumour activities.

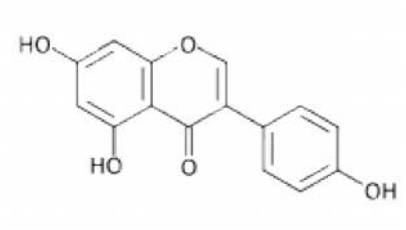
Till there is no study on the combined effect of the silibinin and genestein on the breast cancer and colon cancer. Thus the study was chosen to find out the antitumor activity and antioxidant activity of the two flavonoids in combined and to compare it with the actions of both compound in single.

PLAN OF WORK FOR THE STUDY

1. Collecting the compounds.
2. *Invitro cytotoxicity studies.*
    a. Tetrazolium salt assay
3. *Invitro cytoprotectivity studies*.
    a. Estimation of lipid peroxidation.
    b. Estimation of reduced glutathione.
    c. Estimation of LDH leakage.
4. Results of cytotoxic and cytoprotective activity.
5. Interpretation of the results.
6. Discussion.
7. Conclusion.

COMPOUND PROFILE

GENISTEIN

Genistein	
	
IUPAC name 5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one	
Other names 4',5,7-Trihydroxyisoflavone	
Properties	
Molecular formula	$C_{15}H_{10}O_5$
Molar mass	270.24 g mol^{-1}
Exact mass	270.052823
Meltimg point/freezing point	> 280 °C
STORAGE Provide appropriate exhaust ventilation at places where dust is formed. Protect against light. Store in cool place. Keep container tightly closed in a dry and well-ventilated place .Recommended storage temperature: -20 °C	

Genistein is a phytoestrogen (estrogen-like chemical compound present in plants) that is derived from certain plant precursors by human metabolism. They are naturally occurring chemical constituents that may interact with estrogen receptors to produce weak estrogenic or anti-estrogenic effects. They are composed of a wide group of nonsteroidal compounds similar in structure and function to human estrogens . A conspicuous feature of the chemical structure of phytoestrogens is the presence of a phenolic ring that, with few exceptions, is a prerequisite for binding to the estrogen receptor. For this reason, phytoestrogens can act as weak estrogen agonists, partial agonists, or as antagonists to endogenous estrogens (such as estradiol) and xenoestrogens (including phytoestrogens) with estrogen receptors in both animals and humans. Therefore, working as estrogen mimics, phytoestrogens may either have the same effects as estrogen or block estrogen's effects. There are three major classes of plant chemical compounds that have estrogen-like actions in the body. They are lignans (enterolactone, enterodiol), isoflavones (genistein, daidzein, biochanin A), and coumestans. The two major chemical classes of phytoestrogens found in people's diets are lignans (enterodiol and enterolactone) and isoflavones (daidzein, genistein, and glycitein). Lignans are the main class of phytoestrogens present in Western diets. Genistein is an isoflavone.

BIOLOGICAL EFFECT

Molecular function

Genistein influences multiple biochemical functions in living cells:

- activation of PPARs
- inhibition of several tyrosine kinases
- inhibition of topoisomerase
- direct antioxidation with some proxidative features
- activation of Nrf2 antioxidative response
- stimulation of autophagy
- activation of estrogen receptor beta
- inhibition of the mammalian hexose transporter GLUT1

- contraction of several types of smooth muscles
- modulation of CFTR channel, potentiating its opening at low concentration and inhibiting it a higher doses.

Activation of PPARs

Isoflavones genistein and daidzein bind to and transactivate all three Parxisomal-proliferator activated receptors (PPAR) isoforms, α, δ, and γ. For example, membrane-bound PPARγ-binding assay showed that genistein can directly interact with the PPARγ ligand binding domain and has a measurable Ki of 5.7 mM. Gene reporter assays showed that genistein at concentrations between 1 and 100 uM activated PPARs in a dose dependent way in KS483 mesenchymal progenitor cells, breast cancer MCF-7 cells, T47D cells and MDA-MD-231 cells, murine macrophage-like RAW 264.7 cells, endothelial cells and in Hela cells. Several studies have shown that both ERs and PPARs influenced each other and therefore induce differential effects in a dose-dependent way. The final biological effects of genistein are determined by the balance among these pleiotrophic actions.

Tyrosine kinase inhibitor

The main known activity of genistein is tyrosine kinase inhibitor, mostly of epidermal growth factor receptor (EGFR). Tyrosine kinases are less widespread than their ser/thr counterparts but implicated in almost all cell growth and proliferation signal cascades.

Redox-active - not only antioxidant

Genistein may act as direct antioxidant, similar to many other isoflavones, may alleviate damaging effects of free radicals in tissues.

The same molecule of genistein, similar to many other isoflavones, by generation of free radicals poison topoisomerase II, enzyme important for maintaining DNA stability.

Human cells turn on beneficial, detoxyfying Nrf2 factor in response to genistein insult. This pathway may be responsible for observed health maintaining properities of small doses of genistein. **(Krzysztof Polkowski et. al, 2000)**

Anthelmintic

The root-tuber peel extract of the leguminous plant *Felmingia vestita* is the traditional anthelmitic of the Khasi tribes of India. While investigating its anthelmintic activity, genistein was found to be the major isoflavone responsible for the deworming property. Genistein was subsequently demonstrated to be highly effective against intestinal parasites such as thepoultry cestode *Raillietina echinobothrida*, the pork trematode *Fasciolopsis buski*, and the sheep liver fluke *Fasciola hepatica*. It exerts its anthelmintic activity by inhibiting the enzymes of glycolysis and glycogenolysis, and disturbing the Ca2+ homeostasis and NO activity in the parasites. It has also been investigated in human tapeworms such as*Echinococcus multilocularis* and *E. granulosus* metacestodes that genistein and its derivatives, Rm6423 and Rm6426, are potent cestocides.

Atherosclerosis

Genistein protects against pro-inflammatory factor-induced vascular endothelial barrier dysfunction and inhibits leukocyte-endothelium interaction, thereby modulating vascular inflammation, a major event in the pathogenesis of atherosclerosis.

Cancer links

Genistein and other isoflavones have been identified as angiogenesis inhibitors, and found to inhibit the uncontrolled cell growth of cancer, most likely by inhibiting the activity of substances in the body that regulate cell division and cell survival (growth factors). Various studies have found that moderate doses of genistein have inhibitory effects on cancers of theprostate, cervix, brain, breast and colon. It has also been shown that genistein makes some cells more sensitive to radio-therapy.; although, timing of phytoestrogen use is also important

Genistein's chief method of activity is as a tyrosine kinase inhibitor. Tyrosine kinases are less widespread than their ser/thr counterparts but implicated in almost all cell growth and proliferation signal cascades. Inhibition of DNA topoisomerase II also plays an important role in the cytotoxic activity of genistein. Genistein has

been used to selectively target pre B-cells via conjugation with an anti-CD19 antibody.

Studies on rodents have found genistein to be useful in the treatment of leukemia, and that it can be used in combination with certain other antileukemic drugs to improve their efficacy.

Estrogen receptor - more cancer links

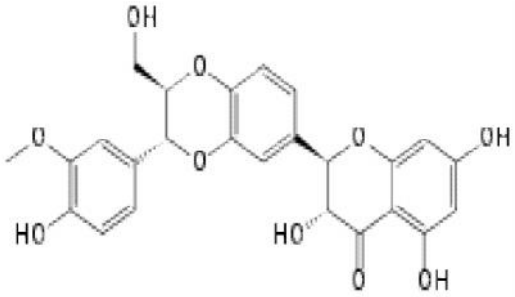
Due to its structure similarity to 17β-estradiol (estrogen), genistein can compete with it and bind to estrogen receptors. However, genistein shows much higher affinity toward estrogen receptor β than toward estrogen receptor α. Data from *in vitro* and *in vivo* research confirms that genistein can increase rate of growth of some ER expressing breast cancers. Genistein was found to increase the rate of proliferation of estrogen-dependent breast cancer when not cotreated with an estrogen antagonist. It was also found to decrease efficiency of tamoxifen and letrozole - drugs commonly used in breast cancer therapy. Genistein was found to inhibit immune response towards cancer cells allowing their survival.

Effects in males

Isoflavones can act like estrogen, stimulating development and maintenance of female characteristics, or they can block cells from using cousins of estrogen. In vitro studies have shown genistein to induce apoptosis of testicular cells at certain levels, thus raising concerns about effects it could have on male fertility; however, a recent study found that isoflavones had "no observable effect on endocrine measurements, testicular volume or semen parameters over the study period." in healthy males given isoflavone supplements daily over a 2 month period. **(Richard A. Dixon, 2004)**

COMPOUND PROFILE

SILIBININ

Silibinin

IUPAC (2*R*,3*R*)-3,5,7-trihydroxy- 2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl) -2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl]chroman-4-one

Properties	
Molecular formula	$C_{25}H_{22}O_{10}$
Molar mass	482.44 g mol^{-1}
STORAGE Store in cool place. Keep container tightly closed in a dry and well-ventilated place. Recommended storage temperature: -20 °C. Avoid contact with skin and eyes. Avoid formation of dust and aerosols.	

Silibinin , also known as silybin, is the major active constituent of silymarin, standardized extract of the milk thistle seeds, containing mixture of flavonolignans consisting of among others of silibinin**,** isosilibinin, silicristin and silidianin. Silibinin itself is mixture of two diastereomers Silibinin A and Silybinin B in approximately equimolar ratio. Both *in vitro* and animal research suggest that silibinin has hepatoprotective (antihepatotoxic) properties that protect liver cells against toxins. Silibinin has also demonstrated anti-cancer effects against human prostate adenocarcinoma cells, estrogen-dependent and -independent human breast carcinoma cells, human ectocervical carcinoma cells, human colon cancer cells, and both small and nonsmall human lung carcinoma cells.

PHARMACOLOGY

Poor water solubility and bioavailability of silymarin led to the development of enhanced formulations. Silipide (trade name Siliphos), a complex of silymarin and phosphatidylcholine (lecithin), is about ten times more bioavailable than silymarin. It has been also reported that silymarin inclusion complex with β-cyclodextrin is much more soluble than silymarin itself. There have also been prepared glycosides of silybin, which show better water solubility and even stronger hepatoprotective effect.

Silymarin, as other flavonoids, has been shown to inhibit P-glycoprotein-mediated cellular efflux. The modulation of P-glycoprotein activity may result in altered absorption and bioavailability of drugs that are P-glycoprotein substrates. It has been reported that silymarin inhibits cytochrome P450 enzymes and an interaction with drugs primarily cleared by P450s cannot be excluded.

BIOLOGICAL EFFECTS

LIVER

Milk thistle prevents toxins from entering the liver by guarding the organ's numerous doorways-the membranes of liver cells. By slowing the rate at which the liver absorbs harmful substances, the toxins are excreted through the kidneys before they can cause liver damage. Much of Milk Thistle's protective effect is due to the flavonoid complex silymarin, which acts as a powerful antioxidant, combining with and thus neutralizing harmful free radicals that result from normal metabolic processes and from the breakdown of toxic substances. At least 10 times as potent as vitamin E, silymarin also helps increase levels of two additional antioxidants, glutathione and superoxide dismutase (SOD). A laboratory study showed that silymarin may increase glutathione content in the liver and intestines by up to 50 percent. Silymarin also increases the activity of SOD in erythrocytes (red blood cells) and lymphocytes (white blood cells) formed in the lymphatic tissue in patients with liver disease.Because silymarin is a potent antioxidant in the stomach and intestines, it may also have a role to play in treating inflammatory conditions such as colitis and ulcers.

CANCER

An antioxidant like silymarin would have anti-cancer effects, the molecular effects of silymarin that give it powerful anti-cancer properties have amazed even the scientific community. In the last few years, researchers have begun to discover exactly why silymarin has such broad anti-cancer properties.Among the most promising cancer fighting strategies that researchers are trying to develop are angiogenesis inhibitors (which stop the proliferation of blood vessels that feed tumors), cell cycle regulators, and selective promoters of cancer cell death. Amazingly, silymarin has been shown to possess all of these abilities. A review of research into silymarin's effects on prostate cancer concluded that silymarin has a huge potential to interfere with many molecular events involved in cancer cell growth, progression, and angiogenesis. One study done in August 2008 indicated that silymarin may inhibit metastasis in prostate cancer. Another study done in

September 2008 identified the strong efficacy of silymarin in prostate cancer prevention and intervention, as reported in previous studies.Because of this you would expect silymarin to have activity against a broad range of cancer types, and an examination of the literature shows that silymarin has impressive effects against prostate, colon, ovarian, skin, lung, breast, and cervical cancers in preliminary studies. In the cases of prostate and ovarian cancer, human clinical trials are currently underway both in the USA and Europe. **(www.Naturalhealthconsults.com)**

TOXICITY

The acute toxicity of silymarin and silybin were investigated by oral and intravenous route in various animal species. No mortality or any signs of adverse effects were observed after silymarin at oral doses of 20 g/kg in mice and 1 g/kg in dogs. The median lethal dose (LD_{50}) after intravenous infusion values are 400 mg/kg in mice, 385 mg/kg in rats and 140 mg/kg in rabbits and dogs. These data demonstrate that the acute toxicity of silymarin is very low.

Similarly, its subacute and chronic toxicity are very low; the compound is also devoid of embryotoxic potential. **(http://www.wisegeek.com/what-are-the-medical-uses-of-silibinin.htm)**

MATERIALS AND METHODS

Silibinin and Genistein was purchased from Sigma Aldrich and constituted in dimethyl sulfoxide (DMSO) solvent as 0,100,150 and 200 mg/mL solutions (containing 0.1% DMSO) and frozen at -37°C until used. Culture media, RPMI 1640, fetal calf serum, antibiotics, trypsin-EDTA, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Breast cancer cells (MCF-7), Human Colon cancer cell line, were obtained from the National center for cell Sciences, Pune (MS) and MCF-10A normal breast cell line, human colon tissue from MGM medical college, Aurangabad. Cells were grown and maintained as monolayers in 25-mm2 tissue culture flasks (Sigma) in RPMI 1640 growth medium, containing 15 mM HEPES, and supplemented with 0.45% (wt/wt) gluose, 5.0% fetal calf serum, and 100 U/mL penicillin plus 100 ug/mL streptomycin.

Cell culture

To assess the cytotoxicity on breast cancer cells and colonl cancer cell line to single and combination treatments with silibinin and genistein, cells were subcultured under 5% CO2 at 37°C for 48 hours to reach 80% confluence.

The cells were harvested by gentle scraping with a cell scraper and resuspended in the medium. From the suspension, 2.5 X 10-4 cells in 200 uL was dispensed into each well of 24-well microtiter plates (MTPs) and cultured under the same conditions described above for 48 hours to allow adherence of the cells.

The supernatants were gently aspirated, and genistein and/or silibinin were added over a range of three cytotoxic concentrations in single and combination treatments. In preliminary studies the 50% inhibitory concentration (IC50) of silibinin 20 ug, and that for genistein was 20 ug. Therefore in the combination treatments, the IC50 of one agent was used against varying concentrations of the other. All treated MTPs were incubated at 37°C in a humidified atmosphere of 5% CO2 in air for a maximum of 72hours.

At each time point, the media were replenished with equal volumes of the appropriate reagent concentration. At 72 hours of incubation, the cells in each MTP well were processed for the 3-(4,5-dimethylthiazol-2- yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt (MTT) assay.

To assess the cytoprotectivity on breast cell line and colonl cell line to single and combination treatments with silibinin and genistein, cells were subcultured under 5% CO2 at 37°C for 48 hours to reach 80% confluence.

The cells were harvested by gentle scraping with a cell scraper and resuspended in the medium. From the suspension, 2.5 X 10-4 cells in 200 uL was dispensed into each well of 24-well microtiter plates (MTPs) and cultured under the same conditions described above for 48 hours to allow adherence of the cells.

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At24and72 hours of incubation, 100 uL of the supernatant from each well was gently aspirated into micro centrifuge tubes and stored at -37°C until assayed for lactate dehydrogenase (LDH) enzyme activity.

MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore the amount of formazan produced is directly proportional to the number of viable cells.

After 72h of incubation, 15 ul of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0c for 4h. the medium with MTT was flicked off and the formed formazan crystals were solubilized in 100 ul of DMSO and then measured the absorbacne at 570 nm using micro plate reader. **(Kinzler et. al. 2007)**

LDH assay

LDH activity was measured by a non-radioactive protocol using the LDH cytotox kit. The LDH assay is based on the release of the cytosolic enzyme, LDH, from cells with damaged cellular membranes. Thus, in cell culture, the course of drug-induced cytotoxicity can be followed quantitatively by measuring the activity of LDH in the supernatant.

The previously frozen supernatant was thawed for LDH determination. Briefly, 100 uL per well of each cell-free supernatant was transferred in triplicates into wells in a 96-well MTP, and 100 uL of LDH-assay reaction mixture (dye-catalyst mixture from the kit) was added to each well. After a 3-hour incubation under standard conditions, the absorbance/optical density of the color generated was read on a Multi scan biochromatic automatic microplate reader at 490 nm.

MDA assay

The formation of MDA in cells was measured by the thiobarbituric acid (TBA) method[8]. Briefly, a 0.5 ml aliquot of the cell suspension was added to 0.5 ml of reaction medium containing TBA (0.375%, *m/V)* and TCA (15%, *m/V)* in 0.25 mol/L HCl. The mixture was heated in boiling water for 15 min, cooled to room

temperature, and centrifuged to remove insoluble materials. Then the mixture was measured at 532 nm using 1,1,3,3-tetraethoxypropane as standard and MDA content was expressed as mmol/L MDA/g protein. Protein content was determined by Lowry's method using bovine serum albumin as standard.

GSH assay

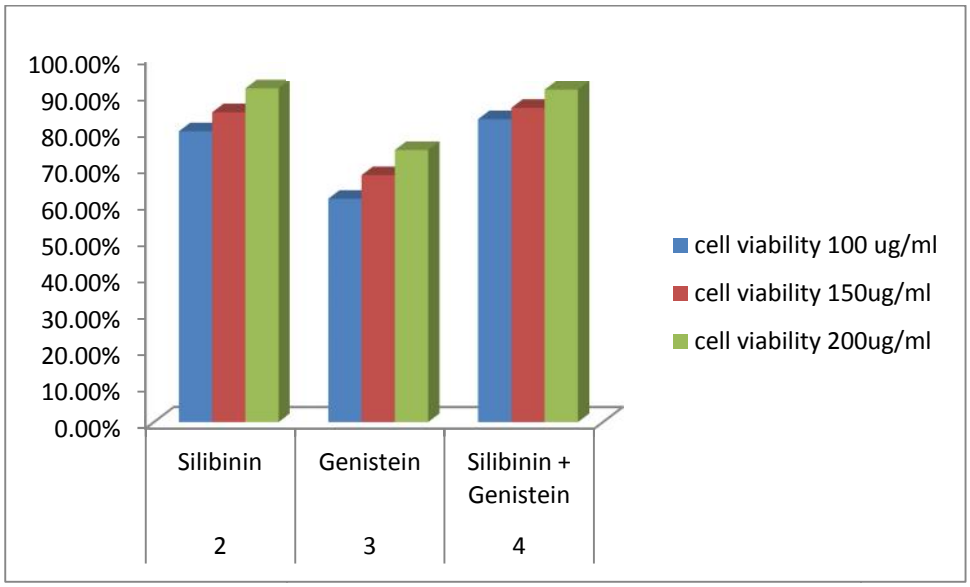
Both Cell Lines were washed and harvested in 0.5 ml of PBS with 0.1% Triton X-100. After 10 min of incubation, the mixture was centrifuged (3000×g, 10 min, 4°C) and 0.3 ml of the supernatant was mixed with 1.0 ml Tris-base (0.8 mol/ L)-EDTA (0.02 mol/L) buffer, pH 8.9. Following the addition of 0.1 ml of DTNB (0.01 mol/L) in methanol[7]. The content of reduced GSH in cells was measured at 412 nm and expressed as mg GSH/g protein. Protein content was also determined by Lowry's method. **(ZHAO Xin-huai,2009)**

RESULTS

Silibinin and Genistein cytotoxicity on Breast Cancer cell line

Sr No	Drugs	Cytotoxicity at three concentrations (%)		
		100 ug/ml	150ug/ml	200ug/ml
1	Control	0%	0%	0%
2	Silibinin	79.375%	84.565%	91.10%
3	Genistein	61.20%	67.66%	74.55%
4	Silibinin + Genistein	83%	86.11%	91.10%

Note : Cytotoxicity in control group was 0. Value were expressed as means of triplicate ± standard deviation



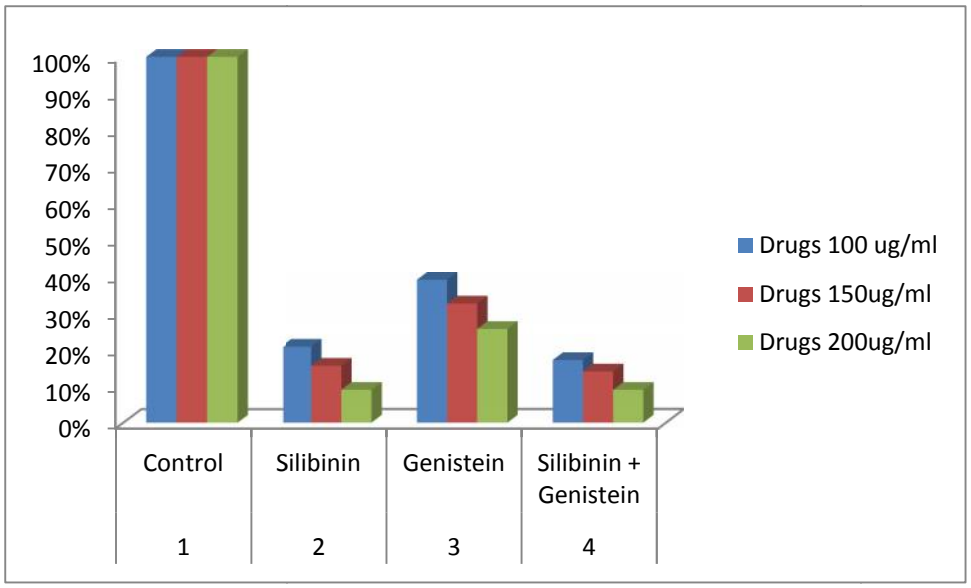
Graph:- Silibinin and Genistein cytotoxicity on Breast Cancer cell line

On Breast cancer cell lines, increase in concentration of the compounds has shown the prominent activity. With the Increase in concentration follows marked inhibition of the growth of cancerous cells. Whereas the combination of the two compounds has shown the very significant activity on the breast cancer cell lines.

Viability of Breast cancer cells in the presence of Silibinin and Genistein.

Sr No	Drugs	Cell viability at three concentrations (%)		
		100 ug/ml	150ug/ml	200ug/ml
1	Control	100%	100%	100%
2	Silibinin	20.625%	15.435%	8.90%
3	Genistein	38.80%	32.34%	25.45%
4	Silibinin + Genistein	17%	13.89%	8.90%

Note : Cell viability in control group was 100. Value were expressed as means of triplicate ± standard deviation



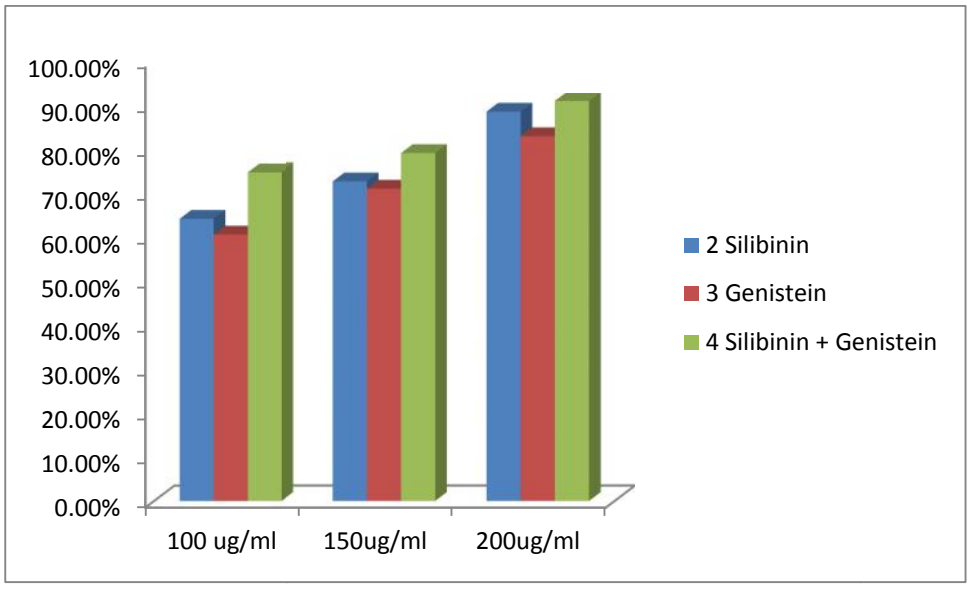
Graph:- Vaibility of breast cells in the persence of silibinin and genistein.

Viability of the breast cancer cells is the (100 – cytotoxicity). In this case as the cytotoxicity goes on increasing the viability of the cells goes on decreasing.

Silibinin and Genistein cytotoxicity on Colon Cancer Cell Lines.

Sr No	Drugs	Cytotoxicity at three concentrations (%)		
		100 ug/ml	150ug/ml	200ug/ml
1	Control	0%	0%	0%
2	Silibinin	64.11%	72.33%	88.11%
3	Genistein	60.54%	70.66%	82.55%
4	Silibinin + Genistein	74.65%	78.74%	90.55%

Note : Cytotoxicity in control group was 0. Value were expressed as means of triplicate ± standard deviation



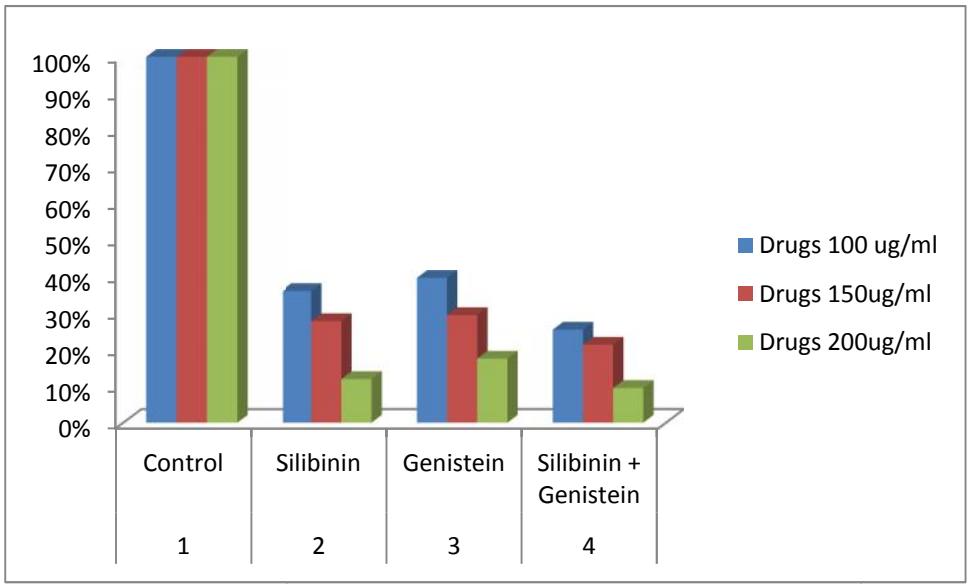
Graph:- Silibinin and Genistein cytotoxicity on Colon Cancer Cell Lines.

On Colon cancer cell lines, both silibinin and genestien hs shown the inhibitory effect. Whereas the combination of the silibinin and genestein has found to be more effective to prevent the growth of the cancerous cell lines. When we are increasing the concentration the activity has also increased in the dose dependent manner.

Viability of Colon cancer cells in the presence of Silibinin and Genistein.

Sr No	Drugs	Cell viability at three concentrations (%)		
		100 ug/ml	150ug/ml	200ug/ml
1	Control	100%	100%	100%
2	Silibinin	35.89	27.67	11.89%
3	Genistein	39.46%	29.34%	17.45%
4	Silibinin + Genistein	25.35%	21.26%	9.45%

Note : Cell viability in control group was 100. Value were expressed as means of triplicate ± standard deviation



Graph:- Vaibility of colon cells in the persence of silibinin and genistein.

Viability of the breast cancer cells is the (100 – cytotoxicity). In this case as the cytotoxicity goes on increasing the viability of the cells goes on decreasing.

Cytoprotective effect of silibinin and Genisteinon Breast cell line.

Sr. No.	Concentration Ug/ml		Viability (%)	LDH	MDA mmol/g	GSH mg/g
1	Control		100	--	0.7 ± 0.2	27.98 ± 0.9
2	H_2O_2			4.3 ± 0.1	2.2 ± 0.2	11.2 ± 0.4
3	Silibinin	100	20.625	4.0 ± 0.2ns	1.8 ± 0.2ns	10.8 ± 0.6ns
		150	15.435	3.1 ± 0.3**	1.3 ± 0.4*	9.3 ± 0.5**
		200	8.90	2.8 ± 0.5**	1.2 ± 0.2**	12.7 ± 0.3**
4	Genistein	100	38.80	3.6 ± 0.2ns	1.6 ± 0.3*	8.9 ± 0.5**
		150	32.34	2.6 ± 0.5**	1.2 ± 0.2**	10.3 ± 0.3ns
		200	25.45	2.8 ± 0.5**	1.2 ± 0.2**	13.1 ± 0.7**
5	Silibinin +Genistein	100	17	3.0 ± 0.4**	1.1 ± 0.3**	10.0 ± 0.4**
		150	13.89	2.7 ± 0.4**	0.9 ± 0.2**	11.2 ± 0.4ns
		200	8.90	2.6 ± 0.4**	1.1 ± 0.5**	12.5 ± 0.4**

Note : Cell viability in control group was 100. Value were expressed as means of triplicate ± standard deviation . The different upper stars represents the significant differences comparing to model group; *($p< 0.05$), ** ($p < 0.01$), ***($p<0.001$).

Cytoprotective effect of silibinin and Genistein on Colon Cell Lines.

Sr. No.	Concentration Ug/ml		Viability (%)	LDH	MDA mmol/g	GSH mg/g
1	Control		100	--	0.7 ± 0.2	27.98 ± 0.9
2	H_2O_2			2.3 ± 0.1	1.7 ± 0.1	13.6 ± 0.8
3	Silibinin	100	35.89	2.3 ± 0.2ns	1.9 ± 0.2ns	9.7 ± 0.4***
		150	27.67	1.4 ± 0.2**	1.1 ± 0.1**	11.3 ± 0.9**
		200	11.89	1.4 ± 0.2**	1.1 ± 0.1**	13.7 ± 0.3ns
4	Genistein	100	39.46	1.9 ± 0.2ns	1.4 ± 0.2ns	11.8 ± 0.6**
		150	29.34	1.3 ± 0.3**	1.1 ± 0.1**	10.7 ± 0.2***
		200	17.45	1.2 ± 0.2**	1.0 ± 0.1***	12.1 ± 0.3**
5	Silibinin +Genistein	100	25.35	1.4 ± 0.3**	1.3 ± 0.2ns	8.8 ± 0.7***
		150	21.26	1.3 ± 0.1**	1.1 ± 0.1**	10.5 ± 1.0**
		200	9.4	1.0 ± 0.2***	1.0 ± 0.2**	10.9 ± 0.3**

Note : Cell viability in control group was 100. Value were expressed as means of triplicate ± standard deviation. The different upper stars represents the significant differences comparing to model group; *($p< 0.05$), ** ($p < 0.01$), ***($p<0.001$).

Statistical analysis

Data were expressed as mean ±standard deviation (SD)of at least three independent assays and subjected to a one- way analysis of variance (ANOVA) using SAS version 9.3 software. *P* values≤0.05 were considered as statistically significant.

Toxicity level was calculated by the following formula:

$$Cytotoxicity\% = 1 - \frac{Mean\ absorbance\ of\ toxicant}{Mean\ absorbance\ of\ negative\ control} \times 100$$

Viability % = 100 - Cytotoxicity %

To diminish test error level, MTT strain was added to some wells without cells and along with other wells, absorbance level was read and ultimately subtracted from whole the absorbance.

DISCUSSION

Silibinin and genistein inhibit growth and proliferation of breast cancer cells and colon cancer cells.

The Silibinin and genistein on inhibition of growth and proliferation of breast cancer cells (BCC) and Colon cancer cells (CCC) were determined by using MTT bioassays. In single and combination treatments, both silibinin and genistein inhibited cell growth and decreased cell survival through induction of cell death in both a time and dose dependent manner. The data obtained revealed that BCC were more sensitive to the combination treatment than to the single treatments ($P < .01$).

For comparative studies, normal (non-cancerous) cells were subjected to identical treatments and bioassay analysis. The results obtained demonstrated the BCC and CCC to exhibit significantly cytotoxicity in response to both silibinin and genistein when compared with the normal cells. In addition to its cytotoxic effect the silibinin and genistein has shown the cytoprotective effect on the normal cell lines. This indicates that the compounds protects the normal host cells and killing the cancerous cells.

The results indicate that both silibinin and genistein have significant inhibitory effect on the growth rate of BCC and CCC. The result also demonstrate that both silibinin and genistein, in single and combination treatments, induced apoptosis in the cancer cells in a time and dose-dependent manner. Growth inhibition effects of both silibinin and genistein have been observed in many human adenocarcinoma cell lines.

The growth inhibition correlated significantly with the degree of treatment induced cell death and induction of apoptosis in the targeted cells. The significant correlation between the two non-radioactive assays (LDH and MDA) justifies the authenticity of the results.

Treatment of BCC with silibinin or genistein result in significant increase in cell death($p<0.01$), indicating a significant cell injury this is same with colon cancer cell line. But when combination is there it is very much significantly work on both.

CONCLUSION

The present investigation was carried out to evaluate the in-vitro cytotoxic and cytoprotective activity of silibinin and genistein on breast and colon cell lines.

It was found that there was significant decrease in the viability of the cells, when the cells are treated with the two compounds silibinin and genistein. The compounds has shown marked cytotoxicity in single and in combination treatments. The compounds has shown a significant results in combination treatment on the breast and colon cancer cell lines comparison with the single treatment.

The antioxidant nature of genistein has confirmed by in-vitro antioxidant studies which showed good results at different concentrarions and also the silibinin has also shown good cytoprotective activity in-vitro in single and in combination treatments.

The results of present investigation proved that the compounds Silibinin and Genistein was found to be effective in inhibiting cancer growth by in-vitro screening The present studies also suggest that the flavonoids are effective and alternative in treatment of tumour and related disorders.

Further studies were essential to characterize and elucidate the detailed mechanism of action of Silibinin and Genistein.

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