

ANTI-PROLIFERATIVE EFFECT OF *SPHAERANTHUS* *AMARANTHOIDES* PLANT EXTRACTS ON COLORECTAL CANCER CELL LINE

Submitted in partial fulfillment of the requirements for
the award of Bachelor of Science degree in Biotechnology

by

KAVIYA R (40770021)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
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This is to certify that this Project Report is the bonafide work of **KAVIYA R** (Register No: 40770021) "ANTI-PROLIFERATIVE EFFECT OF *SPHAERANTHUS AMARANTHOIDES* PLANT EXTRACTS ON COLORECTAL CANCER CELL LINE" under my supervision from November 2020 to May 2023.

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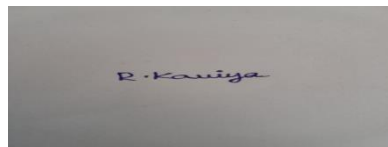
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DECLARATION

I **KAVIYA R** (Register No: 40770021) hereby declare that the project report entitled “**ANTI-PROLIFERATIVE EFFECT OF SPHAERANTHUS AMARANTHOIDES PLANT EXTRACTS ON COLORECTAL CANCER CELL LINE**” was done by me under the guidance of **Dr. S. GRACY JENIFER Ph.D.**, at Department of Biotechnology, Sathyabama institute of science and technology is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in **BIOTECHNOLOGY**.

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ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of **SATHYABAMA** for their kind encouragement in doing this project and for completing it successfully. We are grateful to them.

I convey my thanks to **DR. V. Ramesh Kumar Ph.D.**, Head of the Department of Biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr. S. Gracy Jenifer Ph.D.**, for her valuable guidance, suggestions and constant encouragement paved way for the successful completion of our project work.

I express sincere heartfelt gratitude to **Dr. C. Arulvasu Ph.D.**, Professor, Department of Zoology, University of Madras, Guindy for providing me constant guidance, help and encouragement when I approached him during the course of my project.

I express my sincere gratitude to **Mr. P. Deepakrajasekar, Mrs. J. Vennila, Ms. S. Niranjini, Mr. V. Rajasekar** and **Ms. A. Aarthi** for their constant help and support during my project work at **Unit of Animal Tissue Culture Lab, University of Madras.**

I wish to express my sincere thanks to all my dear friends and teaching and non-teaching staff members of the Department of Biotechnology at the Sathyabama Institute of Science and Technology and the Department of Zoology at the University of Madras, who were helpful in many ways to the completion of the project.

No words can express the deep sense of gratitude to my beloved parents **Mr. Ramalingam** and **Mrs. Balasundhari** for their love, constant encouragement, obstinate sacrifices, sincere prayers and blessings that inspired, motivated.

ABSTRACT

The number of deaths from cancer is increasing daily and is currently the biggest cause of mortality in the world. Plants are the basis of modern medicine and have been used for medical purposes since the start of humanity. *Sphaeranthus amaranthoides*, sometimes called sivakaranthai, is a plant used in folk medicine to cure skin conditions. Traditionally, the plant was used for the treatment of dermatitis, eczema, and acne. In the present study, we extracted crude samples from various parts of the *Sphaeranthus amaranthoides* using various solvents. Subsequently phytochemical constituents were analyzed. The major aim of the studies checks the anticancer properties of different *Sphaeranthus amaranthoides* extracts on human colorectal cancer cells. After exposing crude extracts towards colorectal cancer cells, the percentage of cancer cells viability were decreased when increasing the crude extracts concentration. At the same time, it shows less cytotoxicity towards normal cell line. Among the four different extracts, root extracted using ethyl acetate shows IC₅₀ value at 200 µg/ml when compared to other extracts. Although changes in the cellular and nuclear morphology were analyzed when compared to the control. Also, crude extracts from root prepared by ethyl acetate showed the fragmentation of DNA which confirms the property of apoptotic induction on colorectal cancer cell line. being subjected to the crude extract, the cancer cells had significant changes in cell shape that were indicative of cell cytotoxicity and apoptosis. Compiling all, further studies on the plant's anticancer characteristics and the isolation of its potential component are required to purify in future for its value in cancer treatment.

Keywords: *Sphaeranthus amaranthoides*, anticancer, antiproliferative, colorectal cancer.

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S. No.	ABBREVIATION	EXPANSION
1.	CRC	Colorectal cancer
2.	HT-29	Colorectal cancer cell line
3.	MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
4.	NCI	National Cancer Institute
5.	ICMR	Indian Council of Medical Research
6.	IARC	International Agency for Research on Cancer
7.	°C	Celsius
8.	FBS	Foetal Bovine Serum
9.	DMEM	Dulbecco's Modified Eagle Medium
10.	O ₂	Oxygen
11.	CO ₂	Carbon dioxide
12.	%	Percentage
13.	µg	Micro gram
14.	ml	Micro Litre
15.	W/V	Weight/volume
16.	µl	Mille litre
17.	DMSO	Dimethyl sulphoxide
18.	DNA	Deoxy ribose nucleic acid
19.	PBS	Phosphate buffered saline
20.	IBD	Inflammatory Bowel Disease
21.	PI	Propidium Iodide

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CHAPTER - 1

INTRODUCTION

1.1 INTRODUCTION

Cancer is a multifactorial disease characterized by the uncontrolled proliferation of atypical cells that rapidly multiply, resulting in the death of the affected person (Zhang *et al.*, 2018). The tumour is an abnormal mass of tissue formed by unregulated proliferation and excessive independent and unreasonable cell division (Rajendran, 2009). Neoplasms can be benign when they are slow-division and localized, causing very few troubles for the host, or malignant when they behave the opposite way when they proliferate rapidly, invade the surrounding tissues, colonize distant organs (metastasis), and eventually cause death of the host (Kumar *et al.*, 2009). There are more than a hundred different types of cancer. Cancer is classified into different types based on the organs affected, such as the skin, lungs, breast, prostate, ovary, liver, and skin (WHO, 2017).

The burden of cancer is caused by lifestyles, economically developing countries, physical inactivity, bad diets, rising populations, and the presence of cancer-associated substances in water, food, air, sunlight, and chemicals. Cancer prevalence is also associated with other risk factors like radiation, air pollution, smoking and alcohol use, a high body mass index, obesity, hormones, and inherited mutations (Kumar, 2013). These factors may act together to start or promote carcinogenesis systematically. The genome undergoes dynamic modifications as a result of cancer (Akhtar and Bansal, 2017).

The cancer groups can be divided into broad types. The primary types of cancer are carcinoma, sarcoma, leukemia, myeloma, and central nervous system cancers (Atefeh *et al.*, 2009). Carcinoma, the most frequent type of cancer, occurs in epithelial cells that cover external and internal body surfaces, such as the lung, liver, breast, colon, uterine, prostate, ovary, pancreas, urinary bladder, and skin cancer (Shaikh Mohd, 2016). Sarcomas are cancers that develop in the supporting tissues of the mesodermal layer, including bone, cartilage, fat, tendons, connective tissue, and muscle (Fursule *et al.*, 2006). Melanoma, retinoblastoma, neuroblastoma, and glioblastoma are all cancers that develop from proliferating cells in the ocular retina, neurons, and neural glia (Tamador *et al.*, 2012). Lymphomas

and leukemias, sometimes known as "liquid tumours," develop in the organs that produce lymphoid and blood cells (Kotamkar *et al.*, 2021). Blastoma tumours that develop from embryonic or immature "precursor" cells, children are more likely than older adults to experience a blastoma (Reddy, 2017).

Tumorigenesis in humans is a multi-step process, and each stage reflects the genetic changes that lead to the progressive transformation of healthy human cells into malignant cells (Ratna, 2002). The transforming oncogenes carried by some tumor viruses are similar in base sequence to the proto-oncogenes located in normal mammalian cells. Malignant cells may have mutations in one or more of these proto-oncogenes. It has been demonstrated that some chromosomal rearrangements, which are present in specific cancers, include proto-oncogenes, changing the way these genes' protein products function (Fan and Johnson, 2011).

2. CANCER STATISTICS

Cancer is a leading cause of death worldwide. In 2020, there will be an estimated 10 million new cancer cases and 10 million cancer deaths worldwide (Hyuna *et al.*, 2020). GLOBOCAN estimated that by 2030, 13 million people will die from cancer each year. According to the World Health Organization's (WHO), cancer became the first or second primary cause of death before the age of 70 in 112 of 183 countries and ranked third or fourth in an additional 23 countries in 2019. According to the Indian Medical Research Council (ICMR), more than 17.3 lakh new cases and more than 8.8 lakh deaths are expected by 2020 (ICMR, 2016).

According to estimates, one in five males or females worldwide are being diagnosed, and one in eight men and one in eleven women die from it. Whereas breast, colorectal, lung, cervix uteri, thyroid, corpus uteri, stomach, liver, bladder, and esophagus cancer are the most common types of cancer in women, lung, prostate, colorectal, stomach, liver, bladder, and esophagus cancer are the most common types in men (Lalremmawia *et al.*, 2021).

3. BIOLOGY OF CANCER

Cancer is considered a genetic disease. Every three mutations in DNA can activate oncogenes or inhibit tumorigenesis in humans (Andrea *et al.*, 2010). The

succeeding gene mutation changes the routine of signalling pathways. Although cancer cells develop a degree of self-signalling and promote uncontrolled cell division (Andrea *et al.*, 2021). In cancer, the cell's genome introduces gene mutations that disturb the cellular balance between quiescence and cell division. DNA transmutation causes continuous mutations and provides cells with a tumorigenic nature, which induce metastasis. DNA mutations cause defects in the regulatory circuits, which lead to abnormal cell growth (Kumar *et al.*, 2022).

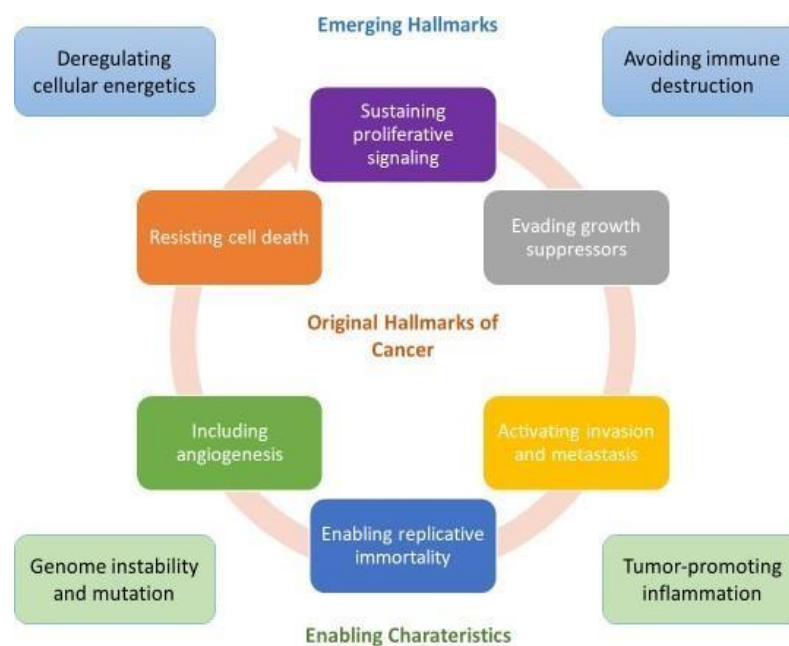


FIG NO. 1.1: THE HALLMARKS OF CANCER

(Source: Hanahan and Weinberg, 2019)

1.4 THE HALLMARKS OF CANCER

The hallmarks of cancer provide knowledge about this illness and a perspective on malignant development and spread (Doru, 2020). Several learnings by cancer cells include self-sustaining growth signals, avoiding growth-inhibitory signals, resistance to apoptosis and immune destruction, metastasis, and expansion of angiogenesis (Dhananjay, 2022). Although irregular utilization of energy metabolism and improper response to immune destruction are the two important budding hallmarks (Rashid & Rashid, 2017). Along with this, the tumour microenvironment promotes an extensive application of these concepts that will be

used to develop a new idea for cancer treatment (Gutschner and Diederichs, 2012). In cancer, mismatched angiogenesis and oxygen delivery, together with the changed metabolism level of cancer cells, all contribute to intratumoral hypoxia (Ming-Zhu and Jin 2020). Sometimes a somatic mutation occurs in some particular DNA. Some of these somatic changes, referred to as “driver mutations,” give a selection advantage for clonal proliferation and are causally connected to the development of cancer (Erin *et al.*, 2010).

1.5 COLORECTAL CANCER

Abnormal cell growth develops in the colon or rectum, resulting in a type of cancer known as colorectal cancer (CRC). The rectum and colon make up the large intestine, sometimes referred to as the "big gut" (Rawla *et al.*, 2019). Precancerous polyps in the colon or rectum are typically the first signs of cancer (Ferlay *et al.*, 2007). Individuals can be diagnosed during yearly screenings or when someone has severe diarrhea or constipation, blood in the stool, stomach pains, or weight loss (William *et al.*, 2009). All colorectal cancer begins with these polyps, but the majority of them do not grow into cancer. Beneficial cells and epithelial cells lining the colon or rectum in the gastrointestinal tract are where colorectal cancer (CRC) originates (Fig No. 1.2) (Boleij & Tjalsma 2012). Colorectal malignant growth is a complex multi-factorial ailment; before the diagnosis processes, there was a wide range of complex hereditary and ecological interactions that have been known to cause malady inception, advancement, and development (Inés *et al.*, 2017).

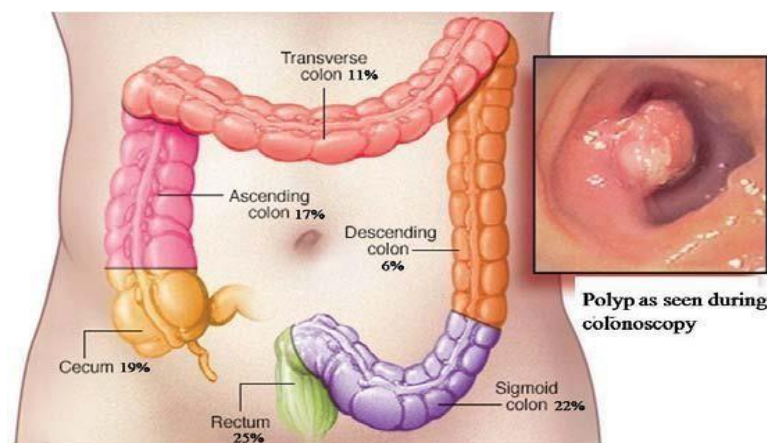


FIG NO. 1.2: INCIDENCE OF CRC IN VARIOUS PARTS OF COLON AND COLONOSCOPY VIEW OF THE POLYP

(Source: Mayo Foundation for Medical Education and Research)

Normal cells of the colonic mucosa are modified into tumor cells through a series of genetic and epigenetic changes, thereby causing the expansion of CRC. The CRC pathogenesis is multiplexed and includes genetic and epigenetic modification. The loss of genomic stability is a crucial molecular stage in the development of cancer. Modifiable and non-modifiable risk factors for CRC are associated with a high incidence. Obesity, food, cigarettes, physical activity, alcohol, and smoking are all modifiable risk factors. Weight, food, smoking use, physical activity, alcohol use, and smoking are all modifiable risk factors. The non-modifiable factors in inflammatory bowel disease (IBD) are adenomatous polyps, ethnicity, genetic inheritance, rising age, and gender. Despite being relatively uncommon, these syndromes offer a thorough mechanistic understanding of all CRC types due to the variety in how they show clinically. It is generally accepted that colorectal cancer has a strong link with genetic variants in nuclear DNA, particularly candidate genes, in a variety of individuals, including the southern populations of India (Fakhria *et al.*, 2022).

1.6 COLORECTAL CANCER STATISTICS

Colorectal cancer ranks third among all cancers in terms of new cases and fourth in terms of mortality (GLOBOCAN, 2020). In India, the number of new CRC cases in men was roughly 40,408 (6.3%), and in women, it was 24,950 (3.7%) of all cancer cases; nonetheless, death rates in Western countries are much lower than in low- and middle-income nations. A total of 19, 31,590 (10%) new colorectal cases and 9, 35,173 (9.4%) deaths were anticipated for all age groups and both genders in 2020. due to the significant geographic diversity in incidence rates around the world (Ferlay *et al.*, 2021). The total number of new CRC cases worldwide is expected to reach 3.2 million in 2040, based on projections of aging, population expansion, and scientific advancement (Xi and Xu, 2021). The second-most common form of cancer in terms of mortality is colorectal cancer (CRC), and it is the third most frequently diagnosed cancer. According to estimates, there will be more than 1.8 million CRC cases and 881,000 CRC-related deaths worldwide in 2018 (Firas *et al.*, 2021).

7. TYPES OF COLORECTAL CANCER

The four main types of colorectal cancer are listed there. These cancer types are adenocarcinomas, carcinoid tumors, gastrointestinal stromal tumors, and sarcomas (Odze *et al.*, 2020). Adenocarcinomas comprise around 95% of all colorectal cancers. These tumors occur in the intestine's glandular cells. These glands produce mucus that lubricates the interior of the colon and the rectum (Mraisel *et al.*, 2018). Carcinoid tumors develop from peculiar hormone-producing intestinal cells (Lips *et al.*, 2003). GIST grow from specialized cells called interstitial cells of Cajal in the colon's wall. But some of them might be benign, and some might be cancerous (Raihana Maqbool, 2016). Sarcomas are originate from blood vessels, muscles, and other connective tissue in the colon's and rectum's walls. Colon and rectal sarcomas are relatively rare (Singh, 2018).

8. STAGES OF COLORECTAL CANCER

Stage describes the extent to which cancer had also spread at the moment of diagnosis. Staging is crucial in determining the most suitable course of treatment and evaluating the prognosis of cancer. The TNM system, also referred to as the American Joint Committee on Cancer (AJCC) staging system, is the most extensively used staging system for colorectal cancer (Jérôme *et al.*, 2014). There are three primary parts: T represents the initial tumour's extent in the intestine's wall and whether it has spread to neighbouring tissues. N indicates how far the tumour has grown into neighbouring lymph nodes. M represents whether the tumour has spread to other body organs, such as the lungs and liver. After the assessment of a patient's T, N, and M status, this data is collected through a procedure known as "stage grouping." The stage is defined by Roman numerals from stage I, the least advanced stage, to stage IV, the most advanced stage. Some stages have letter-based subdivisions (Xiangxing *et al.*, 2018).

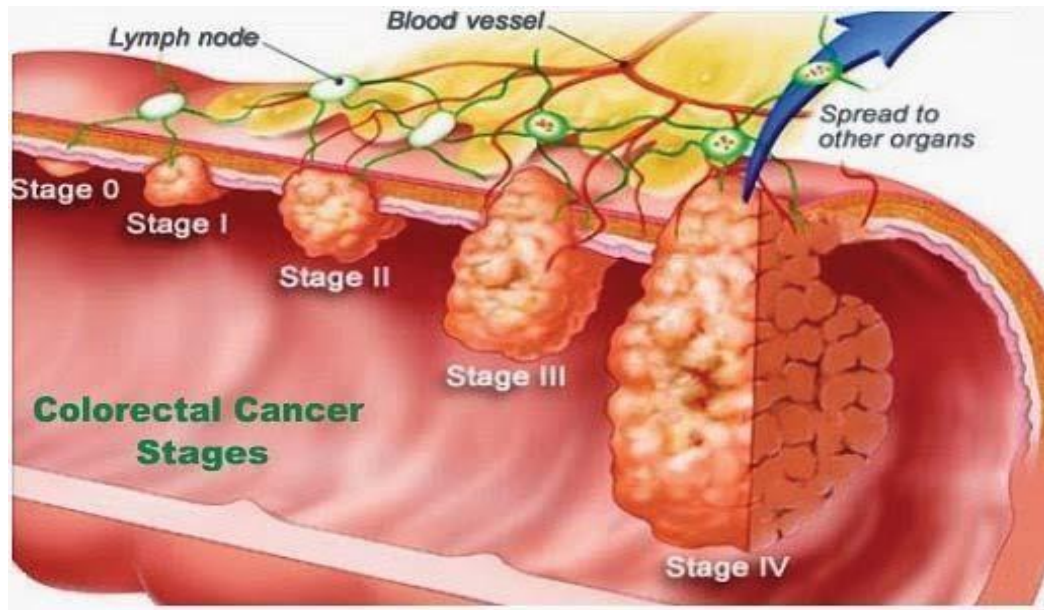


FIG. NO. 1.3: STAGES OF CRC ARE DEPICTED IN A SCHEMATIC

Source: <https://www.ccalliance.org/colorectal-cancer-information/stage-of-diagnosis>

Stage I: cancers that have not yet started to infect the colon or rectum's wall

Stage II: colon cancer or rectum cancers that have expanded into the wall
but haven't passed these to grow to nearby tissues

Stage III: Cancers are those that have progressed through the colon's or
rectum's wall and have affected the lymph nodes or tissues nearby.

Stage IV: Cancers that have moved to the liver or the lung, among other
tissue parts (Fig No.1.3) (Tomita *et al.*, 2021).

1.9 SYMPTOMS

Early-stage CRC is usually without symptoms, but some people with cancer can experience the following signs and symptoms includes irregular bowel habits, Constipation, diarrhoea, or the sensation that the bowels are not empty completely, and blood in the stool that is bright red or very black. Stools that look smaller in size or more narrow than normal, Pain in the abdomen that frequently includes discomfort, bloating, fullness, and gas, diminished appetite or loss of weight, weakness, or exhaustion. Unexplained decreased levels of red blood cells due to iron deficiency anemia (Ratna, 2017).

10. DIAGNOSTICS

In endoscopy the object of equipment with an integrated light is inserted into the body to look for abnormal changes. There are just a few different endoscopic methods for the detection of colorectal cancer. They are colonoscopy, polypectomy, flexible sigmoidoscopy, endoscopic ultrasonography, and barium enema (Gilberto *et al.*, 2019). Flexible sigmoidoscopy is used to look for polyps, cancer, and other abnormalities in the rectum and lower part of the colon. It has been linked to a 21% and 26% reduction in the incidence and mortality of CRC, respectively (Schoen *et al.*, 2012). Colonoscopy is the most accurate way to find polyps or a CRC. A 53% decreased risk of death from CRC was present in patients who had adenomas removed during colonoscopy (with a follow-up colonoscopy at one or three years). Colonoscopy using computed tomography (CT) is less intrusive, has no recovery time, and takes 10 to 15 minutes to complete. Colonoscopy referrals are made for patients with large polyps (>5 mm) or other problematic findings (Zauber *et al.*, 2012).

11. CURRENT THERAPIES FOR COLORECTAL CANCER

There are various CRC treatments available depending on the stage, molecular characterization, and immunological status. Surgery, radiation, chemotherapy, and other kinds of therapy are available for CRC. Surgery refers to the removal of cancer and surrounding tissue. The first treatment option for colon polyps is surgery, also known as surgical removal. Laparoscopic, colostomy, and radiofrequency ablation are procedures used at different stages of CRC. There are several side effects from operations that cause discomfort for the patient (Minggen *et al.*, 2012). The main functional treatment for colon cancer is a colectomy. Surgery is performed to remove all or part of the colon. Hemicolectomy, segmental resection, or partial colectomy are terms used to describe the removal of a part of the colon (Hadrien *et al.*, 2016). A total colectomy is the surgical removal of the entire colon. Abdominoperineal resection (APR) and low anterior resection (LAR) are the two basic surgical treatments for rectal cancer. APR produces an ongoing colostomy by fully removing the distal colon, rectum, and anal sphincter

complex through anterior abdominal and perineal incisions. The upper portion of the rectum is removed during LAR (Iris *et al.*, 2005).

Radiation therapy uses high-energy X-rays to kill cancer cells based on the cancer type, location, and cell size (Jemal *et al.*, 2011). Since radiation therapy is of two types, external beam radiation therapy (EBRT) and internal beam radiation therapy (IBRT), and since each cancer type needed a different approach, radiation therapy was used alone or in combination with other treatment sources. EBRT uses high doses of radiation from an external source, and the equipment allows the radiation beam to fall on the cancerous tissues. It is a local treatment that treats a specific area of the body. Contrastingly, IBRT places the radioactive material in the tumor region of the body with the help of seeds, needles, or capsules. In IBRT, brachytherapy (solid source) and systemic therapies (liquid source) are used based on the source of radioactive material. Radiation treatment therapy for colorectal cancer necessitates accuracy and exactness. Radiation therapy usually consists of treatment given over a specific number of set periods (Raphala *et al.*, 2009).

Chemotherapy is the use of one or more drugs to assassinate the cancer cells, and therapy inhibits cancer's growth. Chemotherapy is given before surgery to shrink the size easy to remove for colorectal cancer in some cases. Chemotherapy and radiation therapy are often given at the same time, called "chemoradiation therapy." Rectal cancer is usually treated with this therapy. Usually, a chemotherapy regimen (schedule) consists of a specific number of cycles given over a specified period. Chemotherapy can be given in several different ways. To reach cancer cells throughout the body, systemic chemotherapy is provided through the bloodstream. The following drugs are currently accepted by the American Food and Drug Administration (FDA) for the treatment of CRC.

Fluorouracil is a kind of systemic chemotherapy used in both secondary and adjuvant treatments as well as analgesic and palliative circumstances. The cornerstone of the CRC treatment is thought to be the fluorinated pyrimidine fluorouracil (5-FU, Aduvicol) (Sobrero *et al.*, 2000). It primarily acts by inhibiting the rate-limiting enzyme in the production of de novo pyrimidine nucleotides, thymidylate synthase. Fluorouracil is usually provided along with leucovorin, a reduced form of folate, which significantly affects its interaction with

thymidylate synthase and increases its capacity for inhibiting DNA synthesis (Sobrero *et al.*, 2000). Capecitabine is substantially more significant than conventional 5-FU. Three enzymatic steps convert it into 5-FU during the digestive tract's absorption process. A further prodrug of fluorouracil used for oral treatment is capecitabine. Mostly through targeted delivery, a crucial enzyme called thymidine phosphorylase (TP), which is thought to be prevalent in tumor tissue at elevated levels, increases this agent's effectiveness and tolerability (Pentheroudakis and Twelves, 2002).

Irinotecan is a semi-synthetic derivative of the natural alkaloid camptothecin (Camptosar, often known as CPT-11). It has a significant role in both first- and second-line alternative treatments for metastatic CRC. It interacts with the enzyme topoisomerase I to produce single-stranded DNA breaks, which have a cytotoxic effect (Ken-ichi, *et al.*, 2015). A novel drug with the potential to treat CRC is oxaliplatin. It has been established to have a significant synergistic relationship with fluorouracil, which produces DNA adducts and induces cellular mortality (Anekant, *et al.*, 2010). Trifluridine/tipiracil is an orally active, novel anti-metabolite drug made of tipiracil, a strong inhibitor of thymidine phosphorylase, and trifluridine, a thymidine-based nucleoside analog (Troy and Uppal, 2016).

Targeted therapy is a type of cancer treatment that targets particular DNA molecules, proteins, or the tissue environment that contributes to cancer growth and survival. This short-term treatment inhibits cancer cell growth and spread while causing minimal damage to healthy cells. A drug having a specific mechanism that specifically inactivates cancer-specific genes, proteins, or biological pathways and causes the malignant process to regress or be destroyed is known as "targeted therapy." Before selecting the appropriate regimen, tests must be performed to determine the targets because these markers are particular to each type of tumor (Michal *et al.*, 2021).

Currently, two promising types of targeted drugs have been presented to treat advanced CRC in clinical trials: Mouse and human monoclonal antibodies against the epidermal growth factor receptor (EGFR) signaling pathway are called cetuximab and panitumumab, respectively. The transmembrane glycoprotein referred to as EGFR is overexpressed in some tumors and is involved in cellular

growth, differentiation, proliferation, and programmed cell death. However, these drugs are only prescribed to patients whose tumors express wild-type RAs, per FDA recommendations. The human monoclonal antibody bevacizumab, which is directed against the vascular endothelial growth factor (VEGF), has also been used in medical procedures to stop angiogenesis (Berlin 2002; Saltz *et al.*, 2004).

12. APOPTOSIS

Apoptosis, additionally referred to as "programmed cell death," is a vital physiological mechanism that regulates a variety of processes, including immunity, homeostasis, getting older, and embryonic development. It is characterized by several morphological abnormalities, including cell shrinkage, membrane blebbing, nuclear chromatin condensation, chromosomal DNA cleavage at internucleosomal locations, and nucleus fragmentation. The plasma membrane's inner side phospholipid serine becomes exposed on its outer layer, where it functions as a recognition signal for phagocyte uptake. Another specific feature that defines apoptosis is the activation of a family of cysteine-aspartic acid proteases called caspases, which causes the cleavage of the nuclear and cytoskeleton scaffolds. Caspases originate as monomeric, inactive proenzymes that are dimerized and frequently cleaved by proteolysis at conserved aspartic residues to become active. They play a crucial role in apoptosis by initiating and concluding it (Bertheloot *et al.*, 2021).

13. MEDICINAL PLANT

The plant is used for medicinal purposes and other studies. Medicinal plants have played an important role in world health since time immemorial. Medicinal plants are important plant species that, according to traditional medicine practices and modern scientific investigations, are used for therapeutic reasons to alleviate ailments and improve human health. The main metabolites that are crucial for the plant's livelihoods, i.e., the plant's machinery, and the secondary metabolites that are not required for plant existence (Babu *et al.*, 2020). Secondary metabolites can protect plants from fungi, bacteria, insects, and viruses. Secondary metabolites extracted from plants have been used as food flavourings, color dyes,

toxins, perfumes, industrial products (e.g., oil), and prescription medications; nevertheless, the activities of the bulk of secondary metabolites are unknown (Madiha *et al.*, 2018).

These bioactive molecules are largely secondary metabolites of plants, and many naturally occurring pure substances have been developed into medications, dietary supplements, and other valuable commercial items (Vijayan, 2011).

Traditional treatments are still used in poor nations. These plants are thought to be rich sources of chemicals that can be employed in medication development and manufacture. Thousands of medicinal plants have been identified as cytotoxic chemicals, providing an extensive number of anticancer medicines and lead compounds. Natural products have been widely employed in the treatment of all chronic diseases, including cancer, and they are also used in acute settings (Tripathi *et al.*, 2005). Ayurveda, an Indian system of medicine, promotes the use of natural substances. Some of them have been tested in various cancer experimental models, both in vitro and in vivo, and have been proven to significantly limit cancer cell proliferation. Natural products are derived from biologically active ingredients. The bioactive components from plant's include alkaloids, flavonoids, tannins, glycosides, steroids, and terpenes (Hassan *et al.*, 2013). The Kalpa medication is essential in the Siddha medical system. The Kalpa medicine mainly used in rejuvenation therapy and eliminates infectious organisms from our bodies and stops them from spreading by triggering an immune response. According to Swarnalatha *et al.*, (2009), the phenolic component in *Sphaeranthus amaranthoides* showed antibacterial and antidiarrheal properties. *Sphaeranthus amaranthoides* is a member of the Kalpa medication class and is a component of some siddha polyherbal formulations with antioxidant properties. In the siddha literature Bhogar reported the plant under the tamil Sivakaranthai, and clearly explain its effect during consumption (Murugesha, 2008).

CHAPTER - 2

LITERATURE SURVEY

Literature showed that our plant sources contain a large number of bioactive compounds and chemicals that are being explored for their anticancer potential to improve our lives easier and better in all ways. *Sphaeranthus amaranthoides* wide range of activities including antimicrobial, anti-inflammatory and antimutagenic activity among them some are tabulated below (Table No. 2.1). One of the ingredients of the authentic herbal supplement medicine "Cansure" is *Sphaeranthus amaranthoides* leaf. It is used to treat advanced cancers of the ovaries, breast, non-small cell lung cancer, and Kaposi sarcoma. The creation of these medications from natural sources has an incalculable impact on chemotherapy. A few other terrestrial medicinal plants having anticancer, antioxidant, and antibacterial properties are list.

The chemical components in plants that induce a particular physiological impact on the human body are what give them their therapeutic value. Alkaloids, saponins, tannins, flavonoids, phenolic compounds, and many more components of these bioactive plant elements are the most important.

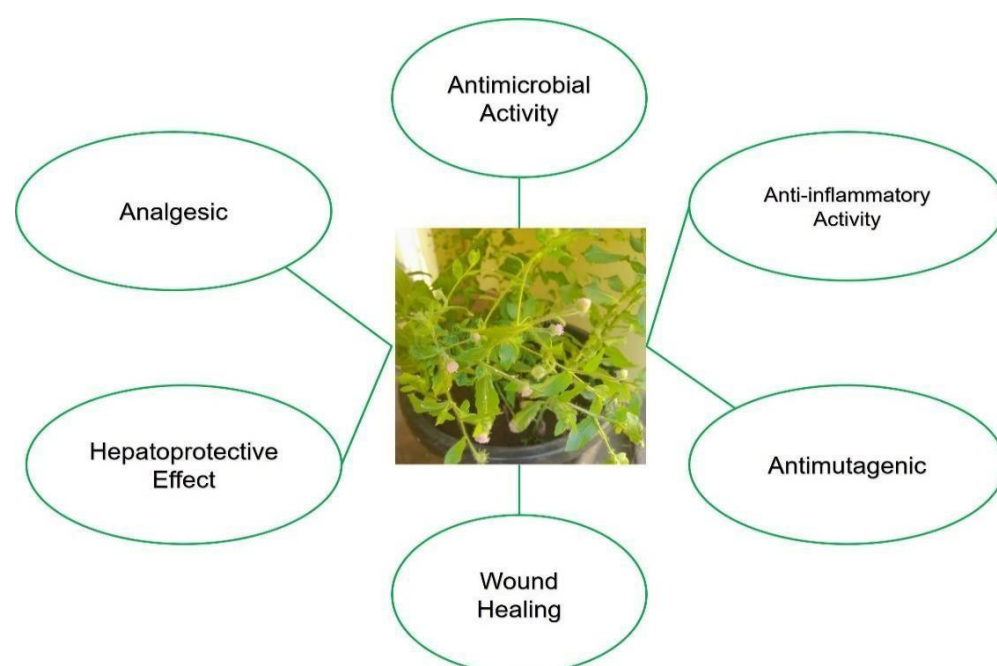


FIG No.2.1: VARIOUS BIOLOGICAL ACTIVITIES OF SPHAERANTHUS AMARANTHOIDES

**Table. No 2.1: ANTICANCER ACTIVITY OF BIOACTIVE COMPOUNDS DERIVED
FROM *SPHAERANTHUS AMARANTHOIDES***

Part of the plant	Solvent used in extraction	Aspect of the study/ cell line used	References
Leaf	Ethanol	Alkaloid fraction Cytotoxicity (A549)	Latha <i>et al.</i> , 2014
Whole plant	Petroleum ether, chloroform, ethyl acetate and methanol	<i>In vitro</i> and <i>in vivo</i> cytotoxicity (Ehrlich's ascites carcinoma)	Gayathri <i>et al.</i> , 2015
Whole plant	Chloroform, ethyl acetate and methanol	<i>In vitro</i> cytotoxicity, Isolation and characterization of secondary metabolites (MCF7, A549, and HT 29)	Gayathri <i>et al.</i> , 2016
Whole plant	Ethanol	Antioxidant, antimutagenic, antimicrobial.	Prabakaran <i>et al.</i> , 2011
Whole plant	Diethyl ether, acetone chloroform and methanol	Antioxidant and wound Healing	Geethalakshmi <i>et al.</i> , 2012

Plant-based medicines can be found easily, are less expensive, safe, and effective, and may only cause minor detrimental consequences. Approximately 80% of people in affluent nations use traditional medicines that contain ingredients derived from medicinal herbs (Martins, 2013). In several Asian nations, plants serve to treat cancer, and beneficial substances from these plants are used in the creation of numerous contemporary anticancer medications. According to recent research, natural products can be used in adjuvant therapy to treat cancer, cure it, and enhance survival of the cancer patients (Esposito *et al.*, 2019). In fact, over the past 50 years, the majority of new clinical applications of secondary plant metabolites and their derivatives have been for treating cancer (Newman *et al.*, 2000; Butter *et al.*, 2004). Drug discovery from medicinal plants has played a significant role in the treatment of cancer.

According to Man *et al.*, (2010), the significant structural diversity of saponins exhibit anti-tumorigenic actions through a variety of anticancer mechanisms. Previous reports statue that alcoholic extract of *Madhuca indica* bark at different concentrations (100, 300, and 500 µg/ml), showed free radical scaveNging activity (Kaushik *et al.*, 2010). Phytochemicals found in the ethyl acetate extract of *Azima tetracantha Lam* leaves showed inhibition of enzymatic activities of phosphodiesterase, phospholipase A2, phosphomono esterase, and acetylcholinesterase enzymes isolated from venom of *Bungarus caeruleus* and *Vipera russelli* (Janardhan *et al.*, 2014). The majority of bioactive substances having reducing and stabilizing agents were derived from plants, algae, bacteria, and fungi (Mittal *et al.*, 2010; Dasmarsili *et al.*, 2010). *Sphaeranthus amaranthoides* is one of them.

In this investigation, colorectal cancer cell lines were employed to test the anticancer activity of crude extracts of *Sphaeranthus amaranthoides* in invitro condition. Based on these studies, the terrestrial medicinal plant *Sphaeranthus amaranthoides* was chosen to produce a potent bioactive substance. There is some evidence in herbal medicine that *Sphaeranthus amaranthoides* is there, the from extract can treat disorders such as oral burns, latex sensitivity, and cancer. It is obvious that terrestrial medicinal plants are a fantastic resource for research and identifying novel chemicals with a diverse spectrum of actions. Thus, a crude extract

obtained from the various parts of the medicinal plant *Sphaeranthus amaranthoides* was selected to check its anti-cancer activity on the HCT116 cell line with the following objectives;

- Preparation of crude extracts from various parts (leaf, stem, and root) of *Sphaeranthus amaranthoides* using various solvents.
- Phytochemical analysis of crude extracts from various parts of the *Sphaeranthus amaranthoides*.
- Screening the cytotoxicity of crude extracts on normal cells line (Vero).
- Screening the cell viability of crude extracts on colorectal cancer cells line (HCT116).
- To study the general and nuclear morphological changes of potentiality active extracts treated colorectal cancer cells line.
- To study the DNA fragmentation analysis of potentially active extract treated colorectal cancer cell line in invitro condition.

CHAPTER - 3

AIM AND SCOPE OF PRESENT INVESTIGATION

1. AIM

The aim of this study to identify various phytochemical constituents, and elucidating the cytotoxicity effect of different crude extracts (methanol, ethyl acetate, hexane, and aqueous,) isolated from various parts of the plant on Vero and human colorectal cancer cell line (HT-29).

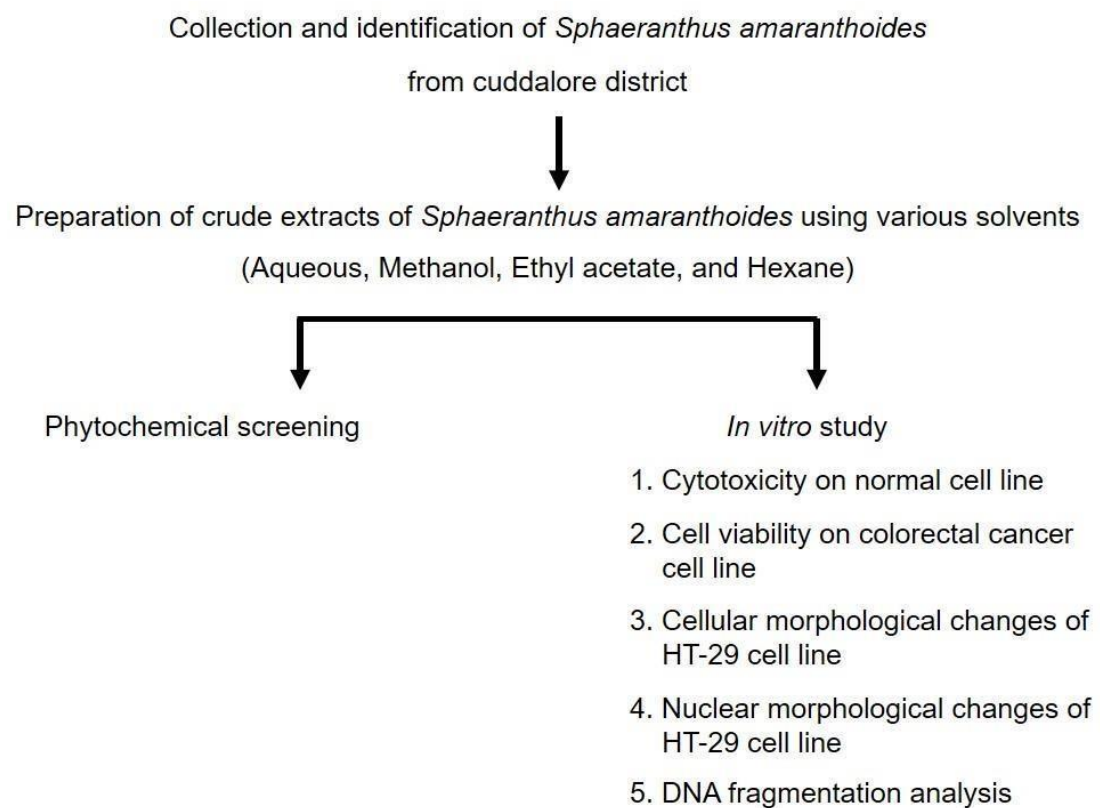
2. SCOPE

To investigate the effect of various crude extracts isolated from various parts of the plant *Sphaeranthus amaranthoides* its phytochemical constituents on normal and cancer cell lines with their cellular and nuclear morphological changes along with the fragmentation of DNA gives a better understanding to use this extract in cancer treatment.

CHAPTER - 4

MATERIAL AND METHODS

4.1 OVERVIEW OF THE STUDY



4.2 COLLECTION AND IDENTIFICATION OF PLANTS

The plant *Sphaeranthus amaranthoides* was collected from the Cuddalore District of Tamil Nadu, India and the plant was identified and authenticated by a botanist from CAS in Botany, University of Madras, Chennai, India.

Nomenclature classification

Kingdom:	Plantae
Phylum:	Angiosperms
Class:	Magnoliopsida
Subclass:	Gamopetalae
Series:	Inferae
Order:	Asterales
Family:	Asteraceae
Genus:	<i>Sphaeranthus</i>
Species:	<i>amaranthoides</i>

4.3 PLANT BIOLOGY

Sphaeranthus amaranthoides is a well-known Indian medicinal plant that belongs to the family Asteraceae. *Sphaeranthus amaranthoides*, known as Sivakarantrhai or Amutakarantai. The genus *Sphaeranthus* in India is represented by three species: *S. indicus*, *S. africanus*, and *S. amaranthoides*. Of these three species, *S. indicus* has been extensively studied (Geethalakshmi *et al.*, 2012).



FIG No. 4.1: COLLECTION SITE (Panruti, Cuddalore, India)



FIG No. 4.2: LEAVES



FIG No. 4.3: STEM



FIG No. 4.4: FLOWER



FIG No. 4.5: DRIED PLANT

4.4 PREPARATION OF CRUDE EXTRACT FROM VARIOUS PARTS OF THE MEDICINAL PLANT

The whole plant of *Sphaeranthus amaranthoides* was collected and washed thoroughly with tap water, followed by distilled water three times. The whole plant is separated into parts (leaf, stem, and root) and air-dried under shade for 5 days. The dried parts were ground into a fine powder using an electric blender. The powder was extracted in the solvents of increasing polarity, viz., hexane, ethyl acetate, and methanol, for 72 hours in the shaker. After it is filtered through Whatman No. 1 filter paper, and kept for evaporation for further use.



Fig No. 4.6 EXTRACTION PROCESS

4.5 PHYTOCHEMICAL SCREENING

The extract was subjected to a preliminary phytochemical screening of its constituents (Kokate, 1988).

Test for alkaloids (Mayer's test):

Mayor's reagent (Potassium mercuric iodide solution) 1.35 grams of Mercuric

chloride was dissolved in 60mL of distilled water. 5 grams of potassium iodide was dissolved in 10mL of distilled water and was made up to 100mL with distilled water.

The extract was dissolved in dilute hydrochloric acid (HCl) and filtered. The filtrate was used to test for the presence of alkaloids. A few drops of reagent were added to 1 ml of extract. The formation of a yellow-cream precipitate indicates the presence of alkaloids.

Test for Cardiac Glycosides (Kella-Killani):

Test the plant extract was dissolved in glacial acetic acid, which contained traces of ferric chloride. Then the tube was held at an angle of 45 degrees, and 1 mL of concentrated sulfuric acid was added down the purple ring at the interface, indicating cardiac glycosides.

Test for Flavonoids:

Alkaline reagent test plant extracts were treated with a few drops of sodium hydroxide solution. The intense yellow color was formed, which became colorless with the addition of dilute acid indicating the presence of flavonoids.

Test for Phenols:

Ferric chloride test to the 1 mL of plant extracts, 3 mL of distilled water was added. To this, a few drops of a neutral 5% ferric chloride solution were added. A dark green color indicated the presence of phenols.

Test for tannins (gelatin test):

The extract (1 ml) and 1% gelatin solution containing sodium chloride (NaCl) have to be added. The formation of a white precipitate indicates the presence of tannins.

Test for Resins:

Acetic anhydride test 1 mL of plant extract, add acetic anhydride solution 1 mL of condensed H_2SO_4 were added. The formation of orange or yellow indicates the presence of resins.

Test for Proteins (Xanthoproteic test):

The extract of 1 ml was mixed with a few drops of concentrated nitric acid (HNO_3). The formation of a yellow colour in the solution indicates the presence of proteins or amino acids.

Test for Resins (Acetic anhydride test):

The 1 ml of plant extract and a few drops of acetic anhydride solution were added. Then add 1 ml of concentrated sulfuric acid (H_2SO_4). The appearance of an orange-to-yellow color indicates the presence of resin.

6. ANTICANCER ACTIVITY OF CRUDE EXTRACTS

1. Cell Line and Chemicals

Colorectal cancer cell lines (HT-29) and normal kidney cell lines (Vero) were obtained from the National Centre for Cell Science (NCCS), Pune, India. Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), sodium bicarbonate, dimethyl sulfoxide (DMSO), and antibiotic solution were purchased from HI Media Laboratories, Mumbai, India. 96-well plates, 6-well plates, tissue culture flasks (25 and 75 mm²), and centrifuge tubes (15 and 50 ml) were purchased from Tarsons Products Pvt. Ltd., Kolkata, India. The chemicals used in the present study were of the highest quality available locally.

Cell culture reagents: Dulbecco's modified Eagle Medium (DMEM): DMEM (3.7 g) was dissolved in 990 ml of milli-Q water. To this solution, 1.5 g of NaHCO_3 and 10 ml of penicillin (100 units/ml)-streptomycin (10 units/ml) cocktail were added and mixed thoroughly, and the pH was adjusted to 7.4 using 1 N NaOH or 1 N HCl. Subsequently, the medium was filtered through a 0.22- μm membrane filter and kept at 4°C. With this, a 10% fetal bovine serum-added medium was used for the maintenance of cultured cells.

Phosphate-Buffered Saline (PBS) pH 7.4:

NaH_2PO_4 (0.63 g), 170 mg of Na_2HPO_4 , and 4.5 g of NaCl were dissolved in 500 ml of distilled water and adjusted to a pH of 7.4 using 1 N NaOH or 1 N HCl

Finally, it was filtered and stored in a sterile container.

Trypsin-EDTA solution:

A trypsin-EDTA solution with a percentage of 0.2 and 0.02 v in PBS was used. The aliquoted trypsin was used and stored frozen until use.

Preparation of growth medium

Ten grams of DMEM were dissolved in 990 ml of sterilized double distilled water. To this solution, 1.5 g of sodium bicarbonate and 10 ml of the penicillin-streptomycin cocktail were added and mixed thoroughly. Later, this medium was filtered using a membrane filter (0.22 μm), dispensed into a sterilized container, and stored at 4°C. FBS (10%) was added to this medium and used for cell culture.

Cell line

An HT-29 cell line derived from colorectal carcinomas showed unusual changes in the chromosome despite the same genetic origin. In the present study, the HT-29 cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India, and maintained in an optimized environment (DMEM medium) supplemented with 10% (V/V) heat-inactivated fetal bovine serum in an atmosphere of 95% air and 5% CO₂. Every passage was made at the confluence of 70–80% trypsinization.

4.6.2 Maintenance of cell lines

Changing Medium

- ❖ The condition of the cells was observed under the inverted microscope. If the colour of the medium became yellow, indicating the medium is acidic, the media was aspirated, and then add fresh media to the flask.

Checking Contamination and Cell Health

- ❖ The flask was removed from the CO₂ incubator and examined under the microscope for contamination (bacterial and fungal) and cross-contamination.
- ❖ The general cell health was checked under the microscope (normal condition of cells) and floating on media.

Subculture or Passage of the Cells

- The flask with 70–80% confluence can be a passage.
- The medium was removed and washed the cells twice with PBS.
- Five hundred microliter of Trypsin (0.1% (w/v)) was added to the flask.
- After 2 to 3 minutes Trypsin was discarded.
- Then the cells were incubated at 37 °C for 30 seconds to 5 minutes.
- Gently, the flask was tapped to loosen all the attached cells.
- Then 5 ml of fresh medium was added to the flask, transferred into a 15 ml centrifuge tube, and centrifuged at 1000 rpm for 5 minutes.
- The pellet was resuspended in 5 ml of medium and distributed to the cells according to the split ratio of the cell line.
- The passage number was maintained. The flask was kept in a CO₂ incubator.

3. Cytotoxicity and cell viability assessment

Cytotoxicity and cell viability were assessed by the MTT technique as described by Mosmann (1983).

Principle:

Rapid colorimetric test for determining the number of viable cells based on the breaking of the tetrazolium ring in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) by dehydrogenases in active mitochondria of living cells.

Materials and chemicals:

1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (5 mg) was dissolved in 1 ml of serum-free DMEM medium.
2. Dimethyl sulfoxide (DMSO)
3. 96-well plates

Procedure:

The normal cell line (Vero) and colorectal cancer (HT-29) were plated in 96-well plates using 5×10^3 cells/ml of DMEM medium with 10% FBS. The cells were allowed to grow for 24 and 28h in a culture medium. To attach cells to the substrates,

the media was removed, rinsed with PBS, and then replaced. Vero cells were then given treatments with various doses of crude extracts (200 to 1400 µg/ml) to assess their cytotoxicity and see if they were safe. Various crude extracts from *Sphaeranthus amaranthoides* were used to analyze the cytotoxicity of normal cells. To evaluate the viability of the colorectal cancer cells with different concentrations such as (50, 100, 150, 200, 250, 300, and 350 µg/ml). The culture plates were incubated After 24hrs Each well was added 50µg of MTT [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] solution and the cells were again incubated for an additional four hours at 37 °C. After adding 100 µl of DMSO, the generated crystals were gently dissolved. The plate was placed on a micro-vibrator for 10 minutes, and an ELIZA reader was used to measure the absorbance at 570 nm.

The following formula is employed to determine the growth inhibition rate:

$$\text{Percentage of Growth inhibition} = \frac{\text{A570 of treated cells}}{\text{A570 of control cells}} \times 100$$

4.6.4 CELLULAR MORPHOLOGICAL OBSERVATION

The general morphological changes in colorectal cancer treated with crude extracts from *Sphaeranthus amaranthoides* Light microscopy were used to examine the cellular morphology of the HT-29 cancer cell line. For that cancer cells (5×10⁴ cells/ml) were DMEM medium containing 10% FBS seeded into six-well plates. The cells were incubated for 24 hours under 5% CO₂ and 95% O₂ at 37°C. After that, the medium was removed, the control plates were given a new medium once more, and the treated plates were given varying concentrations of the crude extracts *Sphaeranthus amaranthoides*. Then the culture plates were incubated as above. After the incubation period, the cells were observed and photographed under a 20X inverted light microscope (Nikon, Eclipse TS 100).

The anticancer activity of crude extracts from various solvents *Sphaeranthus amaranthoides* were tested against a human colorectal cancer cell line. The results showed that the crude extract from root was potentially active against colorectal cancer cell lines while being nontoxic to normal cells. Hence, the crude extract form root was taken to explore further studies.

5. NUCLEAR MORPHOLOGY ANALYSIS

The PI staining method was used in the nuclear morphology analysis procedure described by Rieger *et al.*, (2010).

Principle:

Cells and nucleic acids can be stained with propidium iodide (PI), a cell-impermeant DNA-binding dye. A stoichiometric ratio of one dye per 4-5 base pairs of DNA allows PI to attach to DNA by intercalating between the bases. Sequence preference is observed.

Reagents:

- PI (5 mg) was dissolved in 1 ml of PBS.
- Fixative agents: methanol and acetic acid (3:1 v/v)

Procedure:

Colorectal cancer cells (5×10^3 cells/ml) were plated in a six-well plate containing DMEM medium with 10% FBS. The cells were incubated overnight at 37°C. The culture medium was removed and the fresh medium was given to the control cells, crude extract was given to the treatment wells, and the culture plates were then incubated. After incubation, the incubation cells were washed with PBS, fixed with methanol and acetic acid in a ratio of 3:1 for 10 minutes, and stained with 20 μ l of propidium iodide solution (PI) for 10–20 minutes. The nuclear morphology of cells was immediately observed under the inverted Fluorescent Microscope at 20 x magnifications.

6. DNA fragmentation analysis

DNA extraction and agarose gel electrophoresis were performed by the method of Bortner *et al.*, (1995).

Reagents:

- Lysis buffer (pH 8.0): Tris (12.1 g) and EDTA (7.4 g) were dissolved in 800 ml of double-distilled water; pH was adjusted to 8.0 with 1N HCl; and to this,

8 g of sodium 29 dodecyl sulfate (SDS) was added. Finally, the total volume was made up to 1000 ml.

- Proteinase K: Proteinase K (50 µg) was dissolved in 1 ml of double distilled water.
- Phenol: Chloroform: isoamyl alcohol (25:24:1) Phenol (25 ml), chloroform (24 ml), and isoamyl alcohol (1 ml) were mixed thoroughly.
- Absolute alcohol
- Trisodium acetate: (3 M) trisodium acetate (2.46 g) was dissolved in 10 ml of double-distilled water.
- Ethanol (70%): Ethanol (70 ml) was made up to 100 ml with double distilled water.
- Tris-borate-EDTA (TBE) buffer (pH 8.3): Tris (10.8 g), boric acid (5.5 g), and EDTA (0.93) were dissolved in 70 ml of double distilled water. pH was adjusted to 8.3 with 1 N NaOH, and the final volume was made up to 100 ml with double-distilled water.
- Agarose (1%): agarose (1 g) was dissolved in 100 ml of double-distilled water.
- Ethidium bromide: Ethidium bromide (5 mg) was dissolved in 1 ml of double distilled water.

Procedure:

HT-29 cancer cells (3×10^5 cells/ml) were plated per well in 6-well plates with DMEM medium containing 10% FBS. The cells were incubated for 24 h under 5% CO₂ at 37°C. Then, the DMEM with FBS was removed and washed with PBS. Then the medium was removed, washed with PBS, and fresh serum-free medium was added and kept in an incubator for 1 h. After starvation, the cells were treated with crude extract from root containing medium, and again, culture plates were incubated for 24 hours. After incubation, the DNA was extracted from the cell lysate as follows: The cells were washed with PBS, followed by the addition of 0.5 ml of lysis buffer, and transferred into a microfuge tube. This mixture was incubated for 1 h at 37°C, and to this, 1 ml of proteinase K was added, and incubated at 50°C for 3 h. To each tube, 0.5 ml of h. alcohol (25:24:1) was added, mixed, and centrifuged at 10,000 rpm for 10 min at 4°C. After centrifugation, the supernatant was collected and to

those 2 volumes of ice-cold absolute ethanol, and 1/10 volume of 3 M sodium acetate was added and incubated for 30 min on ice to precipitate DNA. DNA was pelleted by centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was aspirated and the pellet was washed with 1 ml of 70% ethanol. The centrifugation process was repeated until the last traces were removed and the pellet was allowed to dry at room temperature for approximately 30 min and resuspended in 50 µl of TE buffer. 10 g of DNA was electrophoresed in a 1% agarose gel containing ethidium bromide in a mini gel tank containing TBE buffer for 1 h under 90 V, and the gel was examined under a UV transilluminator (Biorad).

4.7 STATISTICAL ANALYSIS

Standard statistical analysis was performed on the data in this study using the SPSS 11.5 statistical software. It was used to find the variation between the control and experimental groups of data. The data appeared as the mean $\pm$ standard deviation of triplicate samples.

CHAPTER 5

RESULT AND DISCUSSION

The medicinal plant *Sphaeranthus amaranthoides* was collected from the Cuddalore district. The *Sphaeranthus amaranthoides* were separated into three main parts (leaves, stem, and root) and dried in the shade. The dried materials were ground into a fine powder. Then, the crude extract was prepared using aqueous and three different solvents, including methanol, ethyl acetate, and hexane, in a ratio of 1:3 (w/v).

5.1 PRELIMINARY PHYTOCHEMICAL SCREENING

The crude extracts of the aqueous, hexane, ethyl acetate, and methanol were first examined for preliminary phytochemicals screening as per the standard protocol. The impact of photochemical constituents is the extracts such as alkaloids, cardiac glycosides, flavonoids, phytosterols, phenols, proteins, resins, tannin, and triterpenes were identified. All of the plant's phytochemical constituents except tannin were present in root part of the plant extracted using methanol as a solvent. All the photochemical constituents except tannin and phenol were present in root part of the plant extracted using ethyl acetate and hexane. The plant parts extracted with water doesn't show any of the phytochemical constituents from the listed one.

TABLE No. 5.1: Preliminary screening of phytochemical constituents in *Sphaeranthus amaranthoides* results

Test	Methanol			Ethyl Acetate			Hexane			Aqueous		
	L	S	R	L	S	R	L	S	R	L	S	R
Alkaloids	+	+	+	+	+	+	+	+	+	-	-	-
Cardiac Glycosides	+	+	+	+	+	+	+	+	+	-	-	-
Flavonoids	-	+	+	-	-	+	-	+	+	-	-	-
Phenol	-	-	+	+	+	-	-	-	-	-	-	-
Phytosterol	-	+	+	-	+	+	-	+	+	-	-	-
Protein	+	+	+	+	+	+	+	+	+	-	-	-
Resins	-	+	+	-	+	+	+	+	+	-	-	-
Tannin	-	-	+	-	-	-	-	-	-	-	-	-
Triterpenes	+	+	+	+	+	+	+	+	+	-	-	-

5.2 CYTOTOXICITY AND CELL VIABILITY OF CRUDE EXTRACTS ON VERO AND COLORECTAL CANCER CELL LINES

The cytotoxicity effect of the crude extracts (methanol, ethyl acetate, hexane, and aqueous) of *Sphaeranthus amaranthoides* was evaluated on the non-cancerous Vero cell line (African green monkey kidney cells) after 24 h of exposure as determined by the MTT assay. The cells were treated with increasing concentrations of (200, 400, 600, 800, 1000, 1200, and 1400 µg/ml). In the medicinal plant parts (leaf, stem, and root), methanol extract till 800 µg/ml, has a percentage of cell viability of 71.01%, 65.89%, and 57.87%; for an ethyl acetate extract, 77.39%, 49.01%, and 57.87%; and for a hexane extract, 42.2%, 55.69%, and 74.1%. At 1400 µg/ml, the percentage of cell viability for medicinal plant components (such as leaf, stem, and root) in an aqueous extract is 69.98%, 6.78%, and 72.29%, respectively. The percentage of cytotoxicity increased with increasing dosages of crude extracts.

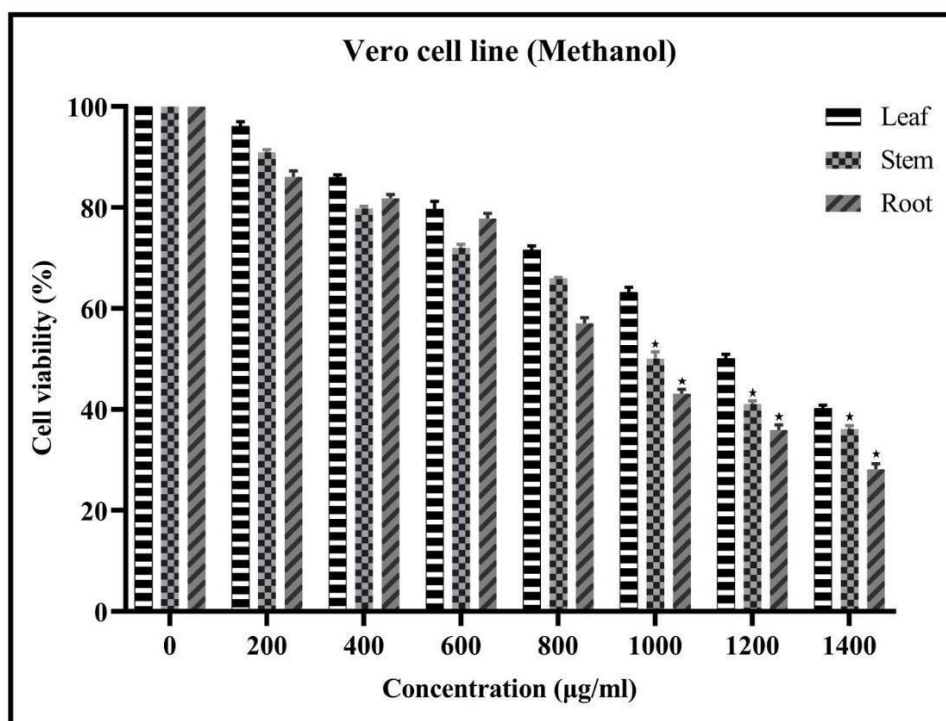


FIG NO. 5.1: CYTOTOXICITY OF METHANOL EXTRACT FROM SPHAERANTHUS AMARANTHOIDES ON VERO CELL LINE FOR 24 HOURS

The data were represented as mean $\pm$ standard deviation of three replicates.
 $P < 0.05$ compared with the control group.

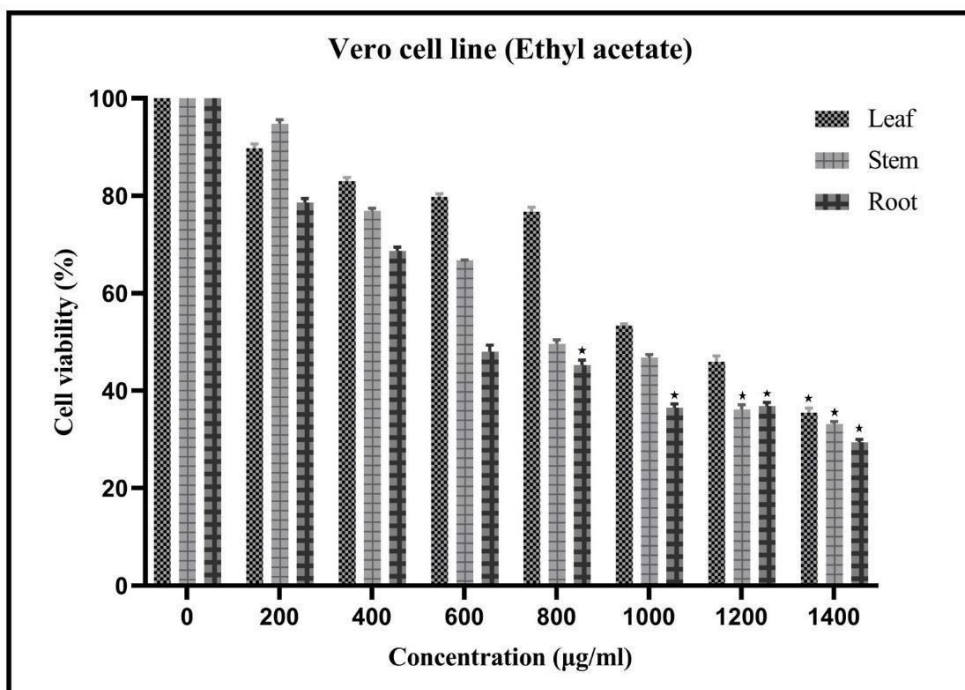


FIG NO. 5.2: CYTOTOXICITY OF ETHYL ACETATE EXTRACT FROM SPHAERANTHUS AMARANTHOIDES ON VERO CELL LINE FOR 24 HOURS

The data were represented as the mean $\pm$ standard deviation of three replicates.
 $P < 0.05$ compared with the control group.

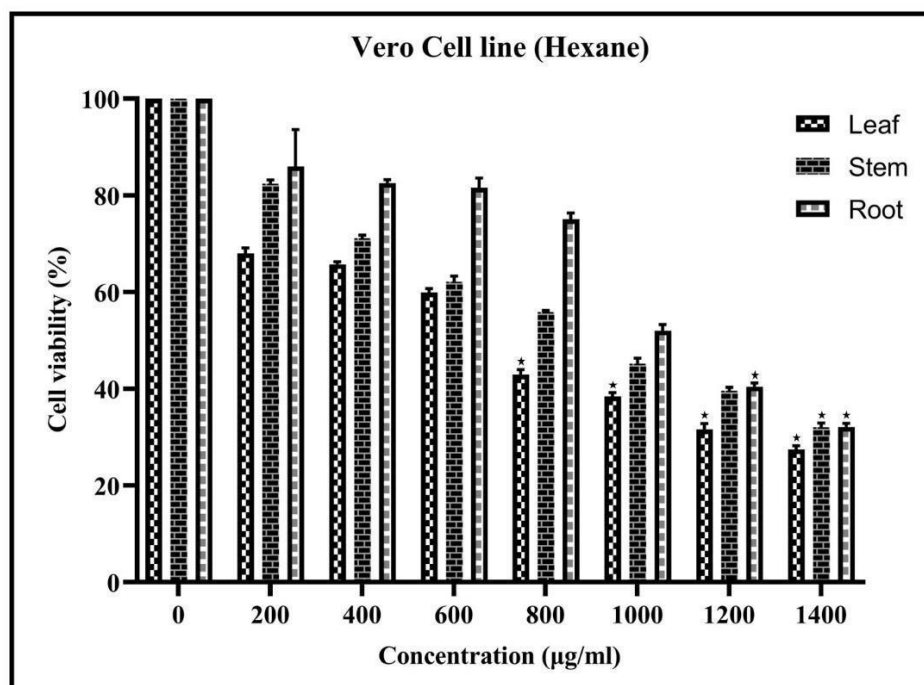


FIG NO. 5.3: CYTOTOXICITY OF HEXANE EXTRACT FROM SPHAERANTHUS AMARANTHOIDES ON VERO CELL LINE FOR 24 HOURS

The data were represented as mean $\pm$ standard deviation of three replicates.
 $P < 0.05$ compared with the control group.

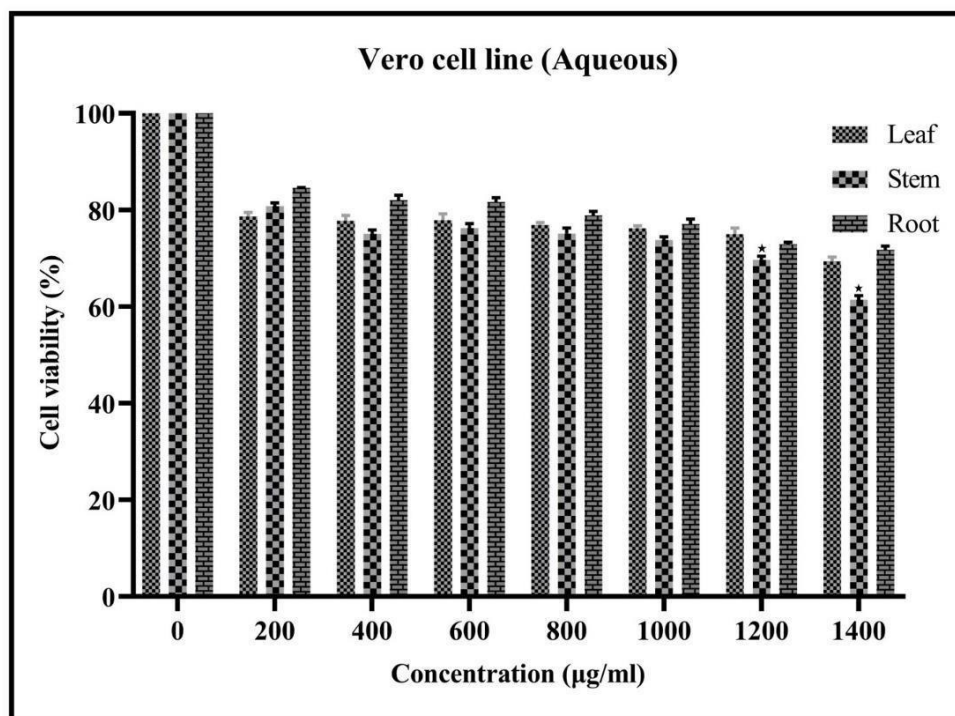


FIG NO. 5.4: CYTOTOXICITY OF AQUEOUS EXTRACT FROM SPHAERANTHUS AMARANTHOIDES ON VERO CELL LINE FOR 24 HOURS

The data were represented as mean $\pm$ standard deviation of three replicates.
 $P < 0.05$ compared with the control group.

The cell viability of the colorectal cancer cell line (HT-29) was treated with *Sphaeranthus amaranthoides* leaf, stem, and root crude extracts at concentrations of 50, 100, 150, 200, 250, 300, and 350 µg/ml. The IC₅₀ value (inhibitory concentration) of *Sphaeranthus amaranthoides* extract in methanol, ethyl acetate, and hexane was determined. The IC₅₀ value of *Sphaeranthus amaranthoides* for the methanol extract was 44.1% at 350 µg/ml; the ethyl acetate stem and root extract showed an IC₅₀ value of 51.17% at 250 µg/ml and 47.41% at 200 µg/ml respectively. Stem extract exhibited 53.19% at 300 µg/ml, 46.68% at 350 µg/ml, and root extract showed 53.38% at 350 µg/ml in hexane.

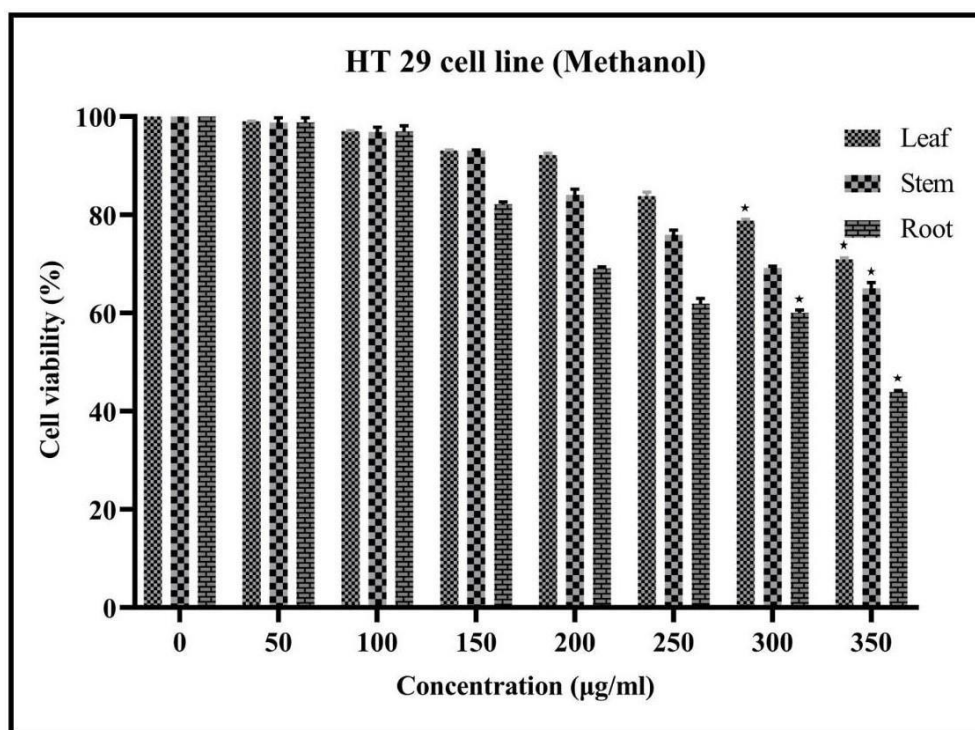


FIG NO. 5.5: CYTOTOXICITY OF METHANOL EXTRACT FROM SPHAERANTHUS AMARANTHOIDES ON HT-29 CELL LINE FOR 24 HOURS

The data were represented as mean ± standard deviation of three replicates.

P < 0.05 compared with the control group.

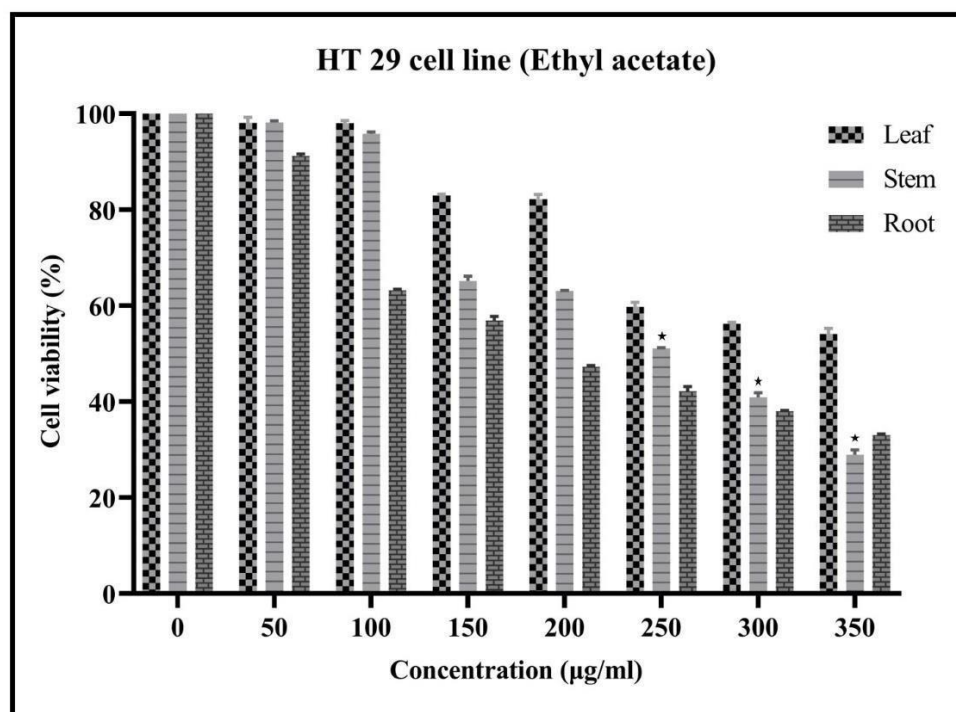


FIG NO. 5.6: CYTOTOXICITY OF ETHYL ACETATE EXTRACT FROM *SPHAERANTHUS AMARANTHOIDES* ON HT-29 CELL LINE FOR 24 HOURS

The data were represented as mean $\pm$ standard deviation of three replicates.
 $P < 0.05$ compared with the control group.

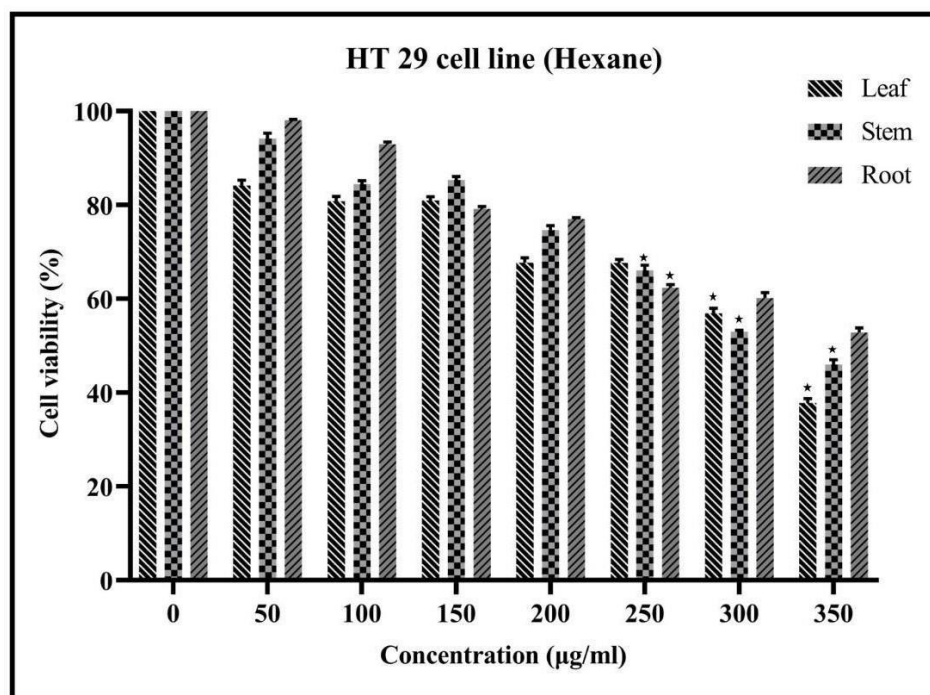


FIG NO. 5.7: CYTOTOXICITY OF HEXANE EXTRACT FROM *SPHAERANTHUS AMARANTHOIDES* ON FROM HT-29 CELL LINE FOR 24 HOURS

The data were represented as mean $\pm$ standard deviation of three replicates.
 $P < 0.05$ compared with the control group.

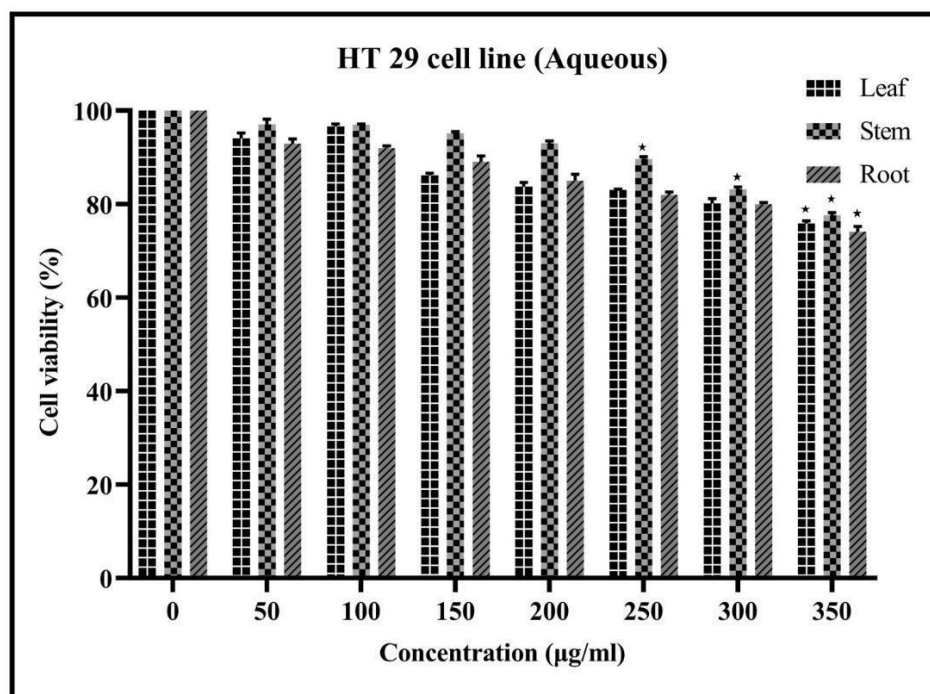
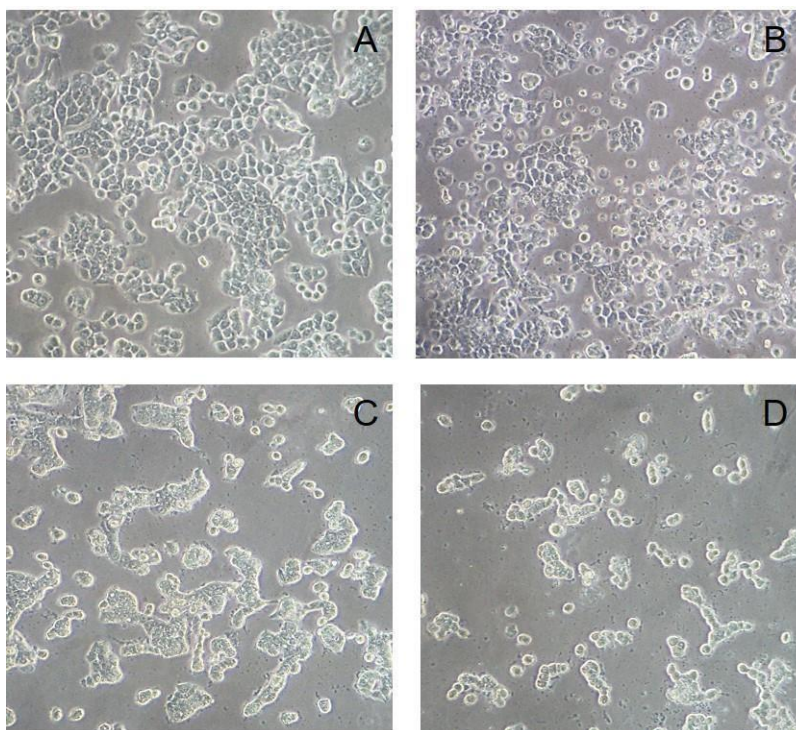


FIG NO. 5.8: CYTOTOXICITY OF AQUEOUS EXTRACT FROM SPHAERANTHUS AMARANTHOIDES ON HT-29 CELL LINE FOR 24 HOURS

The data were represented as mean $\pm$ standard deviation of three replicates.
 $P < 0.05$ compared with the control group

5.3 CELLULAR MORPHOLOGICAL OBSERVATION

A light microscope was used to examine the morphological changes of colorectal cancer cells treated with crude extracts of *Sphaeranthus amaranthoides*. The various concentrations of crude extracts shrink the viability of colorectal cancer cells and cause cellular differentiation. Hence, the three different crude extracts were subjected as control, minimum, IC_{50} , and maximum. The ethyl acetate solvent root part shows to be potentially active against colorectal cancer cells with a minimum delivery of drugs and without damage to normal cells. The HT-29 control cells (without drug) showed uneven confluence, the spherical shape of cells, and even the monolayer. The potentially active crude extract-treated cells at 24 h showed shrinkage and alteration in shape. In contrast, in aqueous extract, every part of the medicinal plant crude extract-treated cells shows no change in cellular shape.



**FIG. NO. 5.9: GENERAL MORPHOLOGICAL CHANGES OF HT-29 CELLS
TREATED WITH POTENTIALLY ACTIVE CRUDE EXTRACTS**

- A: Control cells
- B: Minimum concentration of treated cells (50 µg/ml)
- C: IC₅₀ (200 µg/ml)
- D: Maximum concentration of treated cells (350 µg/ml)

5.4 EXAMINATION OF NUCLEAR MORPHOLOGY OBSERVATION

After being administered treatment with the potentially active crude extract, the HT-29 cells are stained with PI. Because live cells have a vital membrane and absorb less dye, they emit very little fluorescence. Due to their existence and undamaged membranes, control cells emit a very faint amount of fluorescence. The level of fluorescence increases as the concentration does. The cells treated with the lowest concentration had little emission, whereas the cells treated with the highest IC₅₀ showed little emission. The maximum concentration revealed that the emission of fluorescence increased as the live cell count sharply declined.

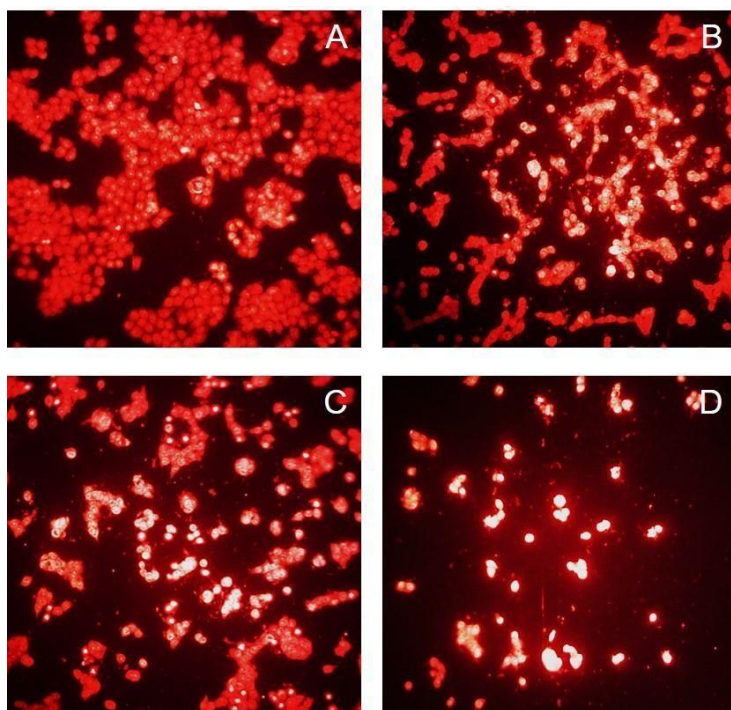


FIG NO. 5.10: NUCLEAR MORPHOLOGICAL CHANGES OF HT-29 CELLS TREATED WITH ETHYL ACETATE USING PI STAINING

- A: Control cells
- B: Minimum concentration of treated cells (50 µg/ml)
- C: IC₅₀ (200 µg/ml)
- D: Maximum concentration of treated cells (350 µg/ml)

5.5 DNA Fragmentation Assay

The ethyl acetate root extract of *Sphaeranthus amaranthoides* was used in a DNA fragmentation experiment to study DNA cleavage, a significant hallmark in apoptosis induction. The *Sphaeranthus amaranthoides* ethyl acetate root extract was applied to the cells at three different concentrations: minimum 50 µg/ml, IC₅₀ 200 µg/ml, and maximum 350 µg/ml. According to the results, the crude extract led to internucleosomal fragmentation in the gastric cell line, which had the impact of causing apoptosis. In contrast with untreated control cells, which showed no DNA fragmentation, agarose gel electrophoresis shows that the intensity of DNA fragmented bands was significant for cells treated with *Sphaeranthus amaranthoides*.

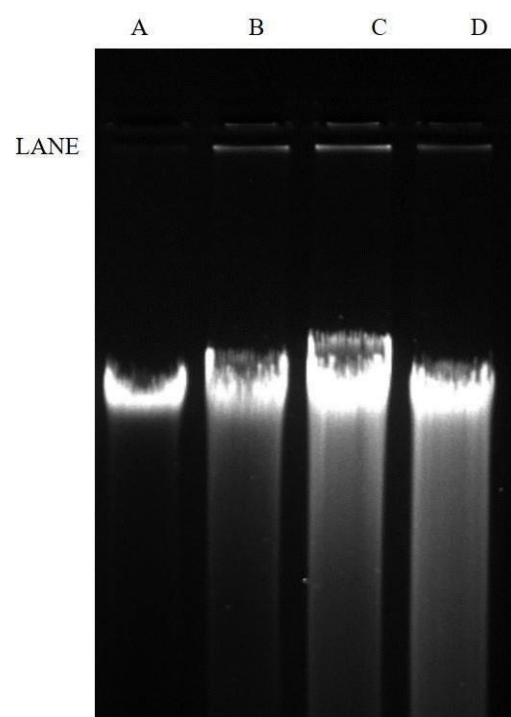


FIG. NO. 5.11: DNA FRAGMENTATION OBSERVATION

A: Control cells

B: Minimum concentration of treated cells (50 µg/ml)

C: IC₅₀ (200 µg/ml)

D: Maximum concentration of treated cells (350 µg/ml)

5.6 DISCUSSION

Natural products have played an important part in every culture and traditional medical system in the world. This has been the case for several millennia (Wangchuk, 2008). However, until the eighteenth century, when they were isolated and identified as distinct individual chemicals with therapeutic potential, bioactive natural products were mostly employed as herbal medicinal preparations (Veeresham, 2012).

Plants have an enormous potential for producing bioactive chemicals that can be used for different kinds of purposes. It was discovered in by Farnsworth *et al.*, 1985 that 119 secondary plant metabolites were employed as medicines. Mostly 11% of the 255 medications can be considered by World Health Organization (WHO), they gave explanation like basic and essential are derived from plants, and

many synthetic medications also start as natural precursors. However, for scientists to verify this traditional claim and advance it to the next stage, clinical trials, molecular characterization, and evaluation are required. *Sphaeranthus amaranthoides* has been the subject of numerous screenings over the past 20 years, including studies on its antioxidant activity (Geethalakshmi, 2013), anti-mutagenic assay (Prabakaran, 2011), analgesic activity (Thanigavelan, 2012), and protective effects on dermal wounds (Swarna Latha, 2009; Sumithra, 2011).

The analysis of the MTT assays demonstrated that root ethyl acetate significantly damages the integrity of the cell membrane and has a cytotoxic effect on the HT-29 cell line in a concentration-dependent manner. This suggests that root ethyl acetate extract affects the development of HT-29 cell lines. Our current study is the first to describe the treatment of normal (Vero) cell lines with a crude extract of *Sphaeranthus amaranthoides* for its cytotoxicity. The analysis of DNA fragmentation and nuclear morphology demonstrates that root ethyl acetate extract has a potent inhibitory impact on cell proliferation and can cause apoptosis in HT-29 cells at low concentrations. According to the findings, bioactive substance from *Sphaeranthus amaranthoides* has the potential to be an anticancer agent. The purification of a potential compound is needed for employing this against cancer treatment.

CHAPTER - 6

SUMMARY AND CONCLUSION

SUMMARY

In the present study crude extracts from various part of the plant *Sphaeranthus amaranthoides* using various solvents were prepared and analysed against colorectal cancer cell line. The medicinal plant was collected from the Cuddalore district, Tamil Nadu, India. The medicinal plant is identified in *Sphaeranthus amaranthoides* parts (leaves, stem, and root) and is dried. The dried parts (Leaf, Stem, and Root) were finely ground and extracted with a few different kinds of solvents (Water, Methanol, Ethyl acetate, and Hexane), then they underwent screening for phytochemical presence. Although several crude extract concentrations were evaluated for cytotoxicity on normal (Vero) cells, up to 75% of cell growth was uninhibited at 600 µg/ml concentration in all the extracts. When the crude extracts were tested for their ability to prevent the growth of colorectal cancer (HT-29) cell lines, it was discovered that ethyl acetate crude extract from the root was the most effective. With very low concentrations, ethyl acetate root extract significantly inhibited colorectal cancer cells (HT-29), and the IC₅₀ value was noted to be reached at 200 µg/ml. In HT-29 cells treated with ethyl acetate root extract at concentrations of 50, 200, and 350 µg/ml, shrinkage of the cells was followed by disruption of cell-cell contact and fragmentation of the nuclei was observed in the cells stained with PI. DNA fragmentation analysis was used to identify the ethyl acetate root extract-induced apoptosis of HT-29 cells. Depending on the exposure dose, DNA fragmentation increased.

CONCLUSION

Over the past couple of centuries, there has been significant progress in the study and development of herbal medicines cancer treatments for encouraged. However, the leading cause of death in the world will continue to be cancer. To lessen the burden of cancer globally, a variety of therapeutic approaches have been employed; however, each of these drugs has drawbacks, including drug resistance,

toxicity, and cost-effectiveness. According to the present research, crude extracts from *Sphaeranthus amaranthoides* has strong anticancer efficacy against gastric cancer.

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