

***IN VITRO* PROPAGATION OF SELECTED
RHODOPHYCEAEN SEAWEED AND EXTRACTION,
CHARACTERIZATION OF ITS SECONDARY METABOLITES
PROJECT WORK**

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

SAHANA SAFRIN A (41300038)

SANDHIYA S (41300040)



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

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

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AICTE**

**JEPPIAAR NAGAR, RAJIV GANDHI SALAI,
CHENNAI - 600 119**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **SAHANA SAFRIN A (41300038)** AND **SANDHIYA S (41300040)** who carried out the project entitled "**IN VITRO PROPAGATION OF SELECTED RHODOPHYCEAN SEAWEED AND EXTRACTION, CHARACTERIZATION OF ITS SECONDARY METABOLITES**" under our supervision from January 2024 to April 2024.

Internal Guide

Ms. JESIA PERSIS PREETHI

External Guide

Dr. L. STANLEY ABRAHAM

Head of the Department

Dr. V. RAMESH KUMAR

Submitted for Viva voce Examination held on 27-04-2024

Internal Examiner

External Examiner

DECLARATION

We, **Sahana Safrin A (41300038)** and **Sandhiya S (41300040)** hereby declare that the Project Report entitled "**IN VITRO PROPAGATION OF SELECTED RHODOPHYCEAEAN SEAWEED, AND EXTRACTION, CHARACTERIZATION OF ITS SECONDARY METABOLITES**" done by us under the guidance of **MS. JESIA PERSIS PREETHI** (Internal Guide) Department of Biotechnology and **Dr.L.STANLEY ABRAHAM** (External Guide) at center of ocean research is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in Microbiology.

DATE: 27-04-2024

PLACE: CHENNAI

Sahana Safrin A.
SIGNATURE OF THE CANDIDATES

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S.	CONTENT	PAGE:NO
N0.	ABSTRACT	IX
	LIST OF ABBREVIATIONS	X
	LIST OF FIGURES	XI
	LIST OF TABLES	XII
1	INTRODUCTION 1	
2	REVIEW OF LITERATURE 13	
	2.1. Major characteristics of seaweed	13
	2.1.1. Green algae-Chlorophyta	13
	2.1.2 Brown algae- Phaeophyta	14
	2.1.3 Red algae- Rhodophyta	15
	2.1.3 General characteristics of seaweed	16
	2.2 Seaweed bioactive components	17
	2.2.1. Phenolic compounds	18
	2.2.2 Proteins	19
	2.2.3 Lipids	19
	2.2.3. Vitamins and minerals	20
	2.2.4 Polysaccharides	21
	2.3. Seaweed extraction	23
	2.4. Anti-oxidant property of seaweed	24
3	AIM AND OBJECTIVES 29	
4	MATERIALS AND METHOD 30	

4.1.1. Preparation of MS media from stock solution	
4.1.2. Inoculation of seaweeds in MS medium	31
v	
4.2. Extraction of secondary metabolites from seaweed	32

.	4.2.1 Soxhlet extraction method	32
	5.3. Purification and screening	
	4.3.1. Column chromatography	33
	4.3.2. Thin layer chromatography	33
	4.4. Molecular characteristics	
	4.4.1. DPPH antioxidant assay	35
	4.4.3. MTT assay for anti-proliferation activity	36
5	RESULT AND DISCUSSION	38
	5.1. Sample collection 5.2:	38
	Seaweed extraction	39
	5.3. Purification and screening	40
	5.4. Molecular characteristics	42
	SUMMARY AND CONCLUSION	
6	REFERENCE	47

ABSTRACT

Cultivating seaweed on a large scale for commercial purposes necessitates a substantial quantity of propagules exhibiting favorable phenotypic traits, including high growth rates and resistance to diseases. The current investigation centered on utilizing marine-based seaweed for in vitro propagation methods, recognized as among the most effective approaches for generating a substantial number of seedlings suitable for commercial cultivation. Crucial factors influencing the growth of in vitro propagation include sterilization techniques, media composition, light intensity, pH, and aeration. Identify the appropriate medium for the extensive-scale production of the micropropagation process. The present project on high-value follows pharmaceutical and nutraceutical products from seaweed and their extraction and characterization of secondary metabolites from seaweed biomass and solvent extraction through Soxhlet extraction and separated using TLC, column chromatography. The separated compounds were extracted for its anti-proliferation properties and anti-oxidant activity

Key words: seaweed, in vitro propagation, inoculation, Soxhlet, light intensity

LIST OF ABBREVIATIONS

1	CC	Column chromatography
2	DPPH	2,2-Diphenyl-1-picrylhydrazyl
3	EAE	Enzyme Assisted Extraction
4	IAA	Indole-3-acetic acid
5	L.digitata	Laminaria digitata
6	L.japonica	Laminaria japonica
7	MAE	Microwave Assisted Extraction
8	MS media	Murashige and Skoog medium
9	MTT	Methyl Thiazolyl diphenyl- Tetrazolium bromide
10	PAA	phenyl acetic acid
11	PGR	Plant growth regulator
12	PSE	Pressure Solvent Extraction
13	SFE	Supercritical Fluid Extraction
14	TLC	Thin layer chromatography
15	UAE	Ultrasound-Assisted Extraction

LIST OF FIGURES

FIGURE NO.	FIGURE NAME	PAGE.NO
2.1	Green algae - Chlorophyta	17
2.2	Brown algae - Phaeophyta	18
2.3	Red algae - Rhodophyta	19
5.1	Sample collection	38
5.2	Gracilaria inoculated on MS media	39
5.3	Soxhlet extraction	39
5.4	Column chromatography (Gracilaria)	40
5.5	Thin layer chromatography (Gracilaria)	41
5.6	DPPH Assay Standard Ascorbic Acid	44
5.7	Gallic Acid Standard	45
5.8	Anticancer effect of sample SA on MCF-7 cell line (graph)	46
5.9	Anticancer effect of sample SA on MCF – 7 cell line	47

LIST OF TABLES

S.NO	TABLES NAME	PAGE NO
1.1	Media Preparation	30
5.1	DPPH Ascorbic Acid Assay Standard	42
5.2	Squalene DPPH Assay	43
5.3	Gallic Acid Standard	43
5.4	Phenolic Content Standard Gallic Acid	44
5.6	Anticancer. effect of sample SA on MCF-7 cell line	45-46

CHAPTER 1 INTRODUCTION

N

Marine algae are fast-growing, very strong photosynthetic organisms with a simple life cycle, also known as marine macroalgae. (Kadam *et al.*,2015). Seaweed are eukaryotic creatures. Seaweeds, often known as ocean algae, are plant species that live in saline water or the ocean. On stony coasts, thick growths frequently occur, as does accumulation in shallow water. The ocean supports key groups of producers, including seaweed (algae) that range in size from microscopic to macroscopic (Joshi *et al.*,2015). These are classified into three major groups: Chlorophyta, Phaeophyceans, and Rhodophyta, which correspond to green, brown, and red algae respectively (Penalver *et al.*,2020). Seaweeds create structural macromolecules called lipids, proteins, and carbohydrates, which are also known as primary metabolites. Countries in East and Southeast Asia dominate seaweed farming and consumption. Between 2000 and 2016, global output of marine macroalgae rose more than thrice, from 10.6 to 32.4 million tonnes (Matos *et al.*, 2021). Approximately 83% of the global seaweed production is commercially sold and consumed directly by humans (Mahadevan, 2015). They grow on solid substrates and plant materials in backwater, shallow, and deep marine settings up to 180 meters deep (Vijayan *et al.*,2016). Seaweeds also include a high concentration of nutrients that are necessary for human nutrition, such as elements, vitamins (K, A, and B12), and vital minerals that act as pigments. Various EU-approved nutritional claims, including those for bone.

This method is utilized to enhance biomass and preserve germplasm, particularly in situations where the population number of seedlings for commercial cultivation. Commercial phenotypic traits, such as a high growth rate and disease resistance. Seaweed has gained much attention over the past decades due to its nutritional and therapeutic benefits. They are considered a good source of protein. Some species of seaweed are known to have protein contents like those of common protein sources like milk, egg, soybean, and meat. Seaweed is considered the sea vegetables and the basis of life in the aquatic habitats, and they have been employed as fertilizer, human food, and animal feed from ancient to Modern times. Seaweeds are commonly exposed to harsh environmental conditions, and the effects of the damage on them are not visible; consequently, the seaweed produces a large range of metabolites (xanthophyllous, tocopherols, and polysaccharides) to protect them from abiotic and biotic factors, such as herbivory and sea mechanical aggression. seaweed metabolites content and diversity are subject to abiotic and biotic factors, such as species, life stages, size,

age, reproductive status location depth, nutrient enrichment, salinity, light intensity exposure, ultraviolet radiation, intensity of herbivory and time of collection. Phenolic compounds are found in seaweed polyphenols synthesized by seaweed, as one of the largest and most widely distributed groups of seaweed phytochemicals, have gained special attention due to their pharmacological activity and array of health-promoting benefits, as polyphenols play a significant role in the high variety of seaweed biological activities. Seaweed phenolic compounds are metabolites that are chemically characterized as molecules containing hydroxylated aromatic rings. This photochemical shows a wide variety of chemical structures, from simple moieties to high molecular polymers. The biogenetically primary synthetic pathways that produce these phytochemicals are the shikimate or the acetate pathway.

Seaweeds are a valuable source of polyphenolic compounds such as phlorotannin, bromophenols, flavonoids, phenolic terpenoids, and cyclosporine-like amino acids. In the brown seaweed seaweeds, the phlorotannin is the major polyphenolic class found only in the marine brown seaweeds. On the other hand, the largest portion of phenolic compounds present in red and green seaweed bromophenols, flavonoids, phenolics acid, phenolic terpenoids, and cyclosporine-like amino acids. These molecules are considered secondary metabolites, as they are protective agents that are produced in response to different stimuli and are defines mechanisms of the seaweeds against herbivory and UV radiation. Phenolic compounds are very difficult to isolate quantitatively at an industrial scale due to their structural similarity and tendency to react with other compounds. However, these compounds possess chemical properties that enable their extraction and purification, allowing highly purified extracts to be obtained at a lab scale. Most phenolic compounds possess a broad variety of biological activities, such as anti-dialecticians- inflammatory, anti- microfilament-viral, anti-allergic, anti-diabetic, antioxidant, anti- photoaging, anti- pruritic, hepatoprotective, hypotension, neuroprotective and anticancer properties. Most of the bioactive compounds are related to the interaction of phenolic compounds with protein. (Yanto *et al.*,2019)

Green seaweed contains a significant quantity of phenolic and flavonoid compounds that can be harnessed for their antioxidant properties. Species such as *Ulva clathrate*, *Ulva compress* (formerly known as *Enteromorpha compress a*), *Ulva intestinalis*, *Ulva linza*, *Ulva flexuous*, *Ulva australis* (formerly known as *Ulva pertusa*), *Caps siphon fulvescens*, and *Chaetomorpha moniliger*a were tested and proven to have high radical scavenging activities. These findings will allow the development of new products in drug, cosmetics, or food industries. Furthermore, the phenolic fraction of *Ulva clathrate* has an anti-tumoral effect and this was also verified in *Ulva flexuous* species. The phenolic extract of the last not

only showed cytotoxicity against breast ductal carcinoma cell line, but also antibacterial activity. This proves the interest of phenol of green seaweeds for human health. In more recent studies, the C. socialist phenolic compounds, such as 2,3,8,9- tetrahydroxy benzo[c]chromen-6-one, 3,4,3',4'tetrahydroxy-1,1'-biphenyl, and cladophorol have been identified with interesting antibacterial activity against methicillin-resistant *Staphylococcus aureus*.

Cytotoxicity Apoptotic activity, Anti-proliferative, Reduces pro-apoptotic molecules, Carcinogenesis reduction Anti-tumour, Promotes the decrease of fasting glucose, Augmentation of oral glucose tolerance, Immunomodulation of intestine microbiome, α glucosidase inhibition Anti-diabetic, Growth inhibition Antiviral, Radical scavenging activity Antioxidant, Multitarget ligands promoting Neuroprotector, and Direct scavenging nitric oxide (NO), Decreasing NO production through the inflammatory signalling cascade - inhibiting the enzymes involved in NO production Anti- inflammatory Seaweed phenolic compounds' mechanisms of action. *Caleurpa spp.* (Chlorophyta) have various flavonoids, such as kaempferol and quercetin. These flavonoids have been correlated with antioxidant activity. *Caulerpa canephora* has the same concentration of the rutin hydrate as the brown seaweed Undrain pinnatifid. Red Seaweeds The ecological function of the phenolic compounds in red seaweeds has been barely investigated, but they probably have multipurpose actions in cell life, as antioxidants, chelating, and defence against herbivory agents, as well as cofactors or hormones. Research on *Ulva porifera* revealed that flavonoidrich extracts under

3 KD a molecular weight promoted the decrease of fasting blood glucose, augmentation of oral glucose tolerance, and protection against liver and kidney injury with reduced.

The protein yield of seaweed per unit area is much higher as compared to the terrestrial soybean, pules legumes, and wheat crops. The extensive use of seaweed of seaweed certain limitation such as the bioavailable, area of production, production capacity, presence of contaminants, seasonal variation, storage, seasonal harvesting, for algal-protein extraction. Seaweed belongs to a diverse and large group of microscopic autotrophs and range from unicellular to multicellular life forms. Seaweed have been widely recognized as an interesting source of bioactive molecules. A great deal of research has been carried out to explore the therapeutic potential of seaweed. Algal protein is extracted using acidic, alkaline, and aqueous method, followed by extraction via precipitation, ultrafiltration, or chromatography. Increasing demand and sustainable supply and availability of high-quality seaweed seedlings

for cultivation is still experiencing many obstacles in SE-Sulawesi. Currently, procurement of seaweed seedlings is mainly from previous culture season which is highly dependent on the local climate and seasonal monsoon (Rama *et al.*,2018). Therefore, potential improvements introduced through the application of in vitro techniques are expected to be even higher. The demand for the raw materials in food and phycocolloids industries is increasing day-by-day. Thus, intensive work on new strain selection and improvement of an efficient mass culture system is clearly needed. For the exploitation of seaweed at the cellular level, constitutes a basic powerful tool (Baweja *et al.*,2009).

The seaweed tissue culture is a relatively, developing area of research with just three decades of development and lags far behind that of higher plants, which got started more than a century ago. The revolution made in plant cell and tissue culture studies has formed the foundation or the genetic engineering of crop plants. Micropropagation using tissue culture methods is recently used for the large-scale propagation of clones with superior traits in several crop species. Furthermore, cell culture offers several advantages for the isolation of mutants over conventional selection procedures using whole plant material. Cell suspensions allow a very large number of cells to be screened simultaneously for a desired trait in a reasonable time frame and reproducible (Priya Sampath *et al.*,2008). In addition, regeneration of plants from callus can result in the recovery of new genetic variants because of the wellknown phenomenon of soma clonal variation (Evans and Sharp 1983). Unlike higher plants, seaweeds show distinct alternation of generations (haploid and diploid)

that can be effectively utilized for genetic improvement. Haploid tissues enable easy detection of mutants, while subsequent chromosome doubling produces fertile individuals and homozygous at all loci, and these provide pure breeding lines for selection and hybridization. The methods employed in seaweed tissue culture are essentially the same as described for higher plant tissue culture. It generally involves the preparation of axenic explants and their culture on a solid agar medium enriched with a range of macro- and micro-nutrients, vitamins, sugars (as carbon sources), and plant growth regulators (auxins and cytokinin). The initial studies carried out from 1980 to 1990 have mostly dealt with fundamental aspects of seaweed tissue. These studies have largely concentrated on the development of basic techniques for routine tissue culture of different seaweeds, with a few attempting to enhance the callus induction rate and growth by using different plant growth regulators and carbon sources in an explant culture medium (Yokoi and Hando, 2002; Yukiya *et al.*, 2004; Rajakrishna Kumar *et al.*,200).

Modern seaweed farming relies heavily on seedlings from natural beds or vegetative cuttings from previous harvests. However, this farming method has some disadvantages, such as

physiological variation in the seed stock and decreased genetic variability, which reduces the growth rate, carrageenan yield, and gel strength of the seaweeds. A new method of seedling production that is sustainable, scalable, and produces many high-quality plantlets is needed to support the seaweed farming industry (Sulistiyan *et al.*, 2012). Recent use of tissue culture and micropropagation techniques in eucheumatoid seaweed production has yielded promising results in increasing seed supply and growing uniform seedlings in large numbers in a shorter time. Several seaweed species have been successfully cultured and regenerated into new plantlets in laboratories using direct regeneration, callus culture, and protoplast culture. The use of bio-stimulants and plant growth regulators in culture media increases the seedling quality even further. Seedlings produced by micropropagation grew faster and had better biochemical properties than conventionally cultivated seedlings. Before being transferred to a land-based grow-out system or ocean nets for farming, tissue-cultured seedlings were recommended to undergo an acclimatization process to increase their survival rate (Dillehay *et al.*, 2008). Regular monitoring is needed to prevent disease and pest infestations and grazing by herbivorous fish and turtles during the farming process. The current review discusses recent techniques for producing eucheumatoid and other valuable seaweed farming materials, emphasizing the efficiency of micropropagation and the transition from laboratory culture to cultivation in land-based or open-sea grow-out systems to elucidate optimal conditions for sustainable seaweed production acclimatization bio-stimulants grow-out micropropagation plant growth regulators seaweed seedlings.

Natural seaweeds are macroalgae that thrive on solid bottom substrates such as rocks, dead corals, pebbles, shells, and other plant materials. The three major groups of macroalgae are green algae (Chlorophyceae), brown algae (Phaeophyceae), and red algae (Rhodophyceae). The use of seaweed in human civilization can be traced back to the Neolithic period, with the discovery of seaweed species remnants at the Monte Verde archaeological site in southern Chile (Erlandson *et al.*, 2015). The discovery provided the most definitive evidence of prehistoric human-seaweed interactions, demonstrating that humans gathered and consumed seaweeds for medicine or food at least 14,500 calendar years ago (Dillehay *et al.*, 2008). Current seaweed farming practices, particularly eucheumatoid cultivation in Southeast Asia, rely considerably on seed stock collected from natural beds. This method, however, has some disadvantages, such as physiological variation in the seed stock and over-exploitation of the donor population (Kim *et al.*, 2017). Another alternative method is to use vegetative cuttings from previous harvests as the seed materials. Despite this, the repeated clonal propagation

reduces genetic variability, resulting in a lower growth rate, carrageenan yield, and gel strength (Sulistiyani *et al.*, 2012). Also, as genetic variability decreases, seaweed's vulnerability to diseases increases, potentially leading to seedling shortages and posing a threat to the seaweed farming industry. To overcome these limitations, scientists attempted to propagate and produce seaweed seedlings in the laboratory using tissue culture and micropropagation techniques, followed by hatchery and sea-based grow-out.

Micropropagation allows for the rapid production of many individuals in a short amount of time, which can then be used as seedlings for land-based cultivation or sea-based farming (Yong *et al.*, 2014). Hurtado and Biter (2007) demonstrated that this method has a high potential for providing seed stock for commercial eucheumatoid seaweed farming in a study in which they used tissue culture to induce callus formation in *Kappaphycus alvarezii*, and then used the regenerated plantlets

as propagules for further growth in a land-based nursery, and then as seedlings for seaweed farming. This approach will not only aid in the conservation of the wild marine algae population, but it will also allow for sustainable seaweed production for commercial purposes. Over the last decade, numerous studies on the use of micro-propagated seedlings for seaweed cultivation have been published, with many of them

emphasizing the importance of cultivating high-quality seedlings as a critical factor in increased seaweed production capacity (Reddy *et al.*, 2017, Rama *et al.*, 2018, Budi Yanto *et al.*, 2019, Cahyani *et al.*, 2020). Tissue culture has been identified as one of the methods for improving seaweed seed quality, producing high-quality seedlings with a faster growth rate than conventionally propagated seedlings (Yong *et al.*, 2014, 2015, Budi Yanto *et al.*, 2019). However, most studies focused on improving micropropagation procedures or enhancing farming practices (Baweja *et al.* 2009, Buschmann *et al.*, 2017, Kim *et al.*, 2017, García-Poza *et al.*, 2020), with little attention paid to the transition from laboratory cultivation to sea-based farming, which is an essential link between high-quality farming material production and long-term farming sustainability. This review examines recent advances in seaweed seedling production and cultivation strategies, emphasizing techniques developed for eucheumatoids or other related seaweed species that could potentially be applied to eucheumatoids. The study also integrates the two vital elements of laboratory seedling production and sea-based growout to address the rising seedling shortage problem in the global seaweed farming industry. The seaweed tissue culture involves the preparation of axenic explants and their culture on a solid agar medium enriched with a range of macro and micro-nutrients, vitamins, sugar (carbon source), and plant growth regulators. The development of a tissue culture

system including, bioprocess engineering of tissue cultures the photobioreactor design, and identification of strategies for secondary metabolite production can expedite the production of valuable compounds from the seaweed. Invitro cell culture technique facilitates the development of new-generation technologies in seaweed cultivation and utilization, that collectively help intensive cultivation of higher-yielding strains, coupled with green processing technologies with controlled production of products of potential commercial applications at competitive rates. Tissue culture of seaweed with considerable interest and optimization to further enhance the economic prospects of seaweed resources. The potential impartments

introduced through the application of the invitro technique are expected to be higher. The demand for raw materials in food and phycocolloid industries is increasing day by day. Thus, intensive work on strain selection and improvement of an efficient mass culture system is clearly needed. For the exploitation of seaweed at the cellular level, plant tissue culture constitutes a basic powerful tool.

The most common PGRs used in recent seaweed tissue culture studies are BAP, NAA, indole-3-acetic acid (IAA), phenylacetic acid (PAA), 2,4- dichlorophenoxyacetic acid (2,4D), kinetin, and zeatin. Most studies have found that IAA, NAA, and BAP are the most effective PGRs for inducing callus in *Kappaphycus* (Sulistiono *et al.*,2012, An and Cam 2015, Mo *et al.*,2020) but PGR application in other genera, such as *Gracilaria* and Sargassum, has also been well established. However, although a combination of 2,4-D and kinetin has been shown to induce callus in *K. alvarezii*, no callus was induced in *Gracilaria changii* under the same conditions (Yeong *et al.*,2014). These findings indicated that callus formation necessitates different biological requirements in addition to the low potential for callus regeneration in *Gracilaria* species. Direct regeneration, rather than indirect regeneration through the callus phase, is preferable for micropropagation in this species (Ramlov *et al.*,2013). (Mo *et al.*,2020) recently reported a study on callus induction in *K. striatus* using a combination of NAA and BAP after sterilization with silver nanoparticles as an effective disinfectant. To date, there have been only a few studies on the induction of callus in *K. striatus*, suggesting that the cultivation of *K. striatus* in solid media poses challenges. Besides, (Uji *et al.*,2016).

Recent studies have previously been conducted to overcome epiphyte problems in commercial seaweed cultivation. For example, it has been discovered that the cultivation period and spore culture method of *Gracilaria* are critical factors in controlling its natural pests (Buschmann *et al.*,2017, Ingle *et al.*,2018). It is also suggested that grazers and amphipods, which feed on the epiphytes, be used to control them (Bannister *et al.*,2019).

Lüning and Pang (2003) proposed a high-density seaweed cultivation method to control the epiphytes infestation problem in rope cultures in the sea and seaweed tank cultures on land. The growth of epiphytic algae will be hampered by the cultivated seaweeds in this method because the cultivated seaweeds absorb near all the light and nutrients. Changing the seasonal rhythms of seaweed cultivation could also aid in epiphyte control (Titlyanov and Titlyanova, 2010). (Mulyaningrum *et al.*, 2019) proposed a method for controlling light intensity, which promotes the growth of cultivated seaweeds while reducing the presence of epiphytic algae. Another simple but effective method of controlling epiphytes is to apply mechanical movement to the culture lines, preventing epiphyte propagules or spores from attaching at the cultivated seaweed and cultivation facilities (Mulyaningrum *et al.*, 2019). In terms of protecting seaweeds from grazing by other organisms, which is frequently followed by ice-ice disease, the floating cage cultivation method, as used in *K. alvarezii* and *E. denticulatum* farming, is promising. This protection system allows for seaweed cultivation with modifications based on farming area topography, as has been done in several cultivation areas throughout the Philippines (Ganesan *et al.*, 2006). It is hoped that this mitigation technique can assist farmers in resolving pest and disease issues while remaining productive in their seaweed farming activity.

Seaweed cultivation, particularly eucheumatoids, has progressed considerably over the last ten years. Researchers are incorporating technologies and innovations that take the conventional farming method to the next level, from lab-based cultivation to open-sea farming. In the present study, following lab-scale micropropagation, acclimatization to the outdoor environment is crucial to prepare the seedlings for the grow-out process on the actual farm. During the acclimatization process, the seaweeds are increasingly exposed to environmental stress, such as salinity and temperature variations, as well as direct spectral irradiance. Many studies have shown that acclimatization improves tissue-cultured seaweeds' survival and growth rate during the grow-out period. According to (Yong *et al.*, 2015), micropropagated

K. alvarezii seedlings that were acclimatized in an outdoor tank grew faster in the field ($3.91 \pm 0.16\% \text{ d}^{-1}$) than non-acclimatized seedlings ($3.56 \pm 0.07\% \text{ d}^{-1}$) and were more disease resistant. The tissue culture and acclimatization were found to improve seedling growth and survival rates during the grow-out and farming stages. Farmers now have a better alternative to farming seaweed, especially in extreme weather conditions, with recent land-based seaweed growing system developments. While land-based cultivation studies have provided several groundbreaking options, their integration into conventional farming is still in its early

stages. Further research may be needed to validate the cost-effectiveness of the invented technologies and new techniques in the seaweed cultivation industries. However, based on the promise of the recent development, we anticipate a global revolution in seaweed seedling production in the coming years.

CHAPTER 2

LITERATURE REVIEW

2.1. MAJOR CHARACTERISTICS OF SEAWEED

Seaweeds refer to any large marine benthic algae that are multicellular, *macrothallic* and thus differentiated from most algae that are of microscopic size. Seaweeds are large marine benthic algae, that are multicellular, macro thallic, and thus differentiated from most algae that are tiny in size (Ishakani *et al.*, 2016.). Seaweeds are classified into three types: Chlorophyceae (green algae), *Phaeophyceae* (brown algae), and *Rhodophyceae* (red algae). Seaweed categorization is based on color, cellular structure, and several other features. This comprises red, brown, and green algae. (joshi *et al.*,2015). Brown algae (division Phaeophyta) are the most common seaweeds in temperate and polar climates, with their dark color masking the green chlorophyll.

They develop at depths of 50-75 feet (15-23 meters). Red seaweeds (division Rhodophyta) may be found in depths of up to 879 ft/268 m. Their red color allows them to absorb blue and violet light (Meena *et al.*,2010). There are around 4,000 kinds of green algae. Green algae could be seen in Some species thrive on damp soil, while others prefer coastal or freshwater settings. These algae exist in three types: unicellular, colonial, and multicellular. Examples of green algae include sea lettuce (*Ulva* sp.).

2.1.1. GREEN ALGAE-CHLOROPHYTA

Green algae can be unicellular (one cell), multicellular (many cells), colonial (living in a loose aggregation of cells), or coenocytic (made up of one big cell without cross- walls; the cell might be uninucleate or multinucleate). They have membrane-bound chloroplasts and nuclei. Most green are aquatic and often found in freshwater (mostly charophytes) and marine settings (primarily chlorophytes); some are terrestrial, growing on soil, trees, or rocks. Polysaccharides comprising sulfated fucose, xylans from green seaweeds, and cellulose are present in all genera,

albeit at lower amounts than in higher plants. Green algae, unlike red and brown algae, contain considerable

amounts of palmitidonic (16:4-3) and palmitolinolenic (16:3-3) PUFAs. The chlorophyta cell walls are largely composed of sulfated polysaccharides (Lahaye & Robic, 2007). The majority of Chlorophyta species contain C16 and 18 polyunsaturated fatty acids (PUFA), notably linoleic acid (LA; C18:2-6). In contrast, Ulvales is distinguished by ALA (C18:3-3) (Pereira *et al.*, 2012).



Fig: 2.1. Green algae-Chlorophyta

2.1.2 BROWN ALGAE- PHAEOPHYTA

Brown algae class Phaeophyceae are a group of around 1,500 algae species in the Chromophyta division that are found in cold waters along continental coastlines. The species' color ranges from dark brown to olive green, depending on the ratio of brown pigment (fucoxanthin) to green pigment (chlorophyll). Brown algae differ in shape and size from microscopic filamentous epiphytes (Ectocarpus) to sophisticated gigantic kelps ranging in size from 1 to over 100 meters. Seaweeds are well-known for their high polysaccharide, mineral, and vitamin content. Brown seaweeds have low protein content, while red seaweeds tend to have higher levels. Lipid levels are typically low. (joshi *et al.*, 2015) Brown seaweed belongs to the Phaeophyceae group and is named after its greenish brown pigment, fucoxanthin. Brown seaweeds generate a variety of chemicals, including secondary metabolites such as alkaloids, terpenoids, and phenolics, which have commercial value and unique biological activity. (Ashish *et al.*, 2016). Bioactive sulfated

polysaccharides derived from brown seaweeds have received a lot of attention in pharmacology and biochemistry. Brown seaweeds include functional polysaccharides such fucans and alginic acid derivatives that have anticoagulant, anti- inflammatory, antiviral, and antitumoral effects (Wijesingh *et al.*,2012).



Fig:2.2 Brown Algae-the Phaeophyta

2.1.3 RED ALGAE- RHODOPHYTA

Red algae (division Rhodophyta) are any of over 6,000 species of mostly marine algae that are commonly seen connected to other beach plants. Their morphology includes filamentous, branching, feathery, and sheetlike thalli. The group's taxonomy is disputed, and the Rhodophyta division may not adequately reflect its members' phylogeny. Red seaweeds provide a high protein content, like that of high-protein foods. They have a unique amino acid pattern that complements most terrestrial veggies. More study is needed to assess the nutritional value of marine algae, including proper biochemical analysis and bioavailability assessments. (joshi *et al.*,2015). Red seaweeds contain numerous phenolic compounds such as flavonoids and phlorotannin's, with flavonoids having three linked rings and phlorotannin's having eight or more, making them more effective and stable antioxidants. The phenolic compounds of red seaweed are being researched for many industrial sectors, such as medicinal and cosmetic, because of their strong antioxidant capacity (Sara Grace *et al.*,2020) The most important pigment in red seaweeds is phycocyanin. These chemicals are being used commercially as natural colours in chewing gum, soft drinks, dairy products, and cosmetics such as lipstick and eyeliner.

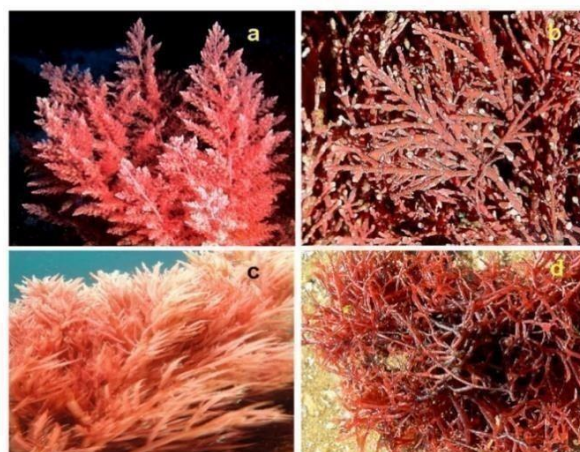


Fig: 2.3 Red Algae-the Rhodophyta

2.2 GENERAL CHARACTERISTICS OF SEAWEED

Macroalgae, sometimes known as seaweed, are multicellular creatures made up mostly of carbohydrates. This feature, with the low or no lignin concentration, makes them intriguing for bioethanol or biobutanol synthesis. (Pabola *et al.*, 2020). Seaweeds are a varied collection of multicellular, non-flowering, plant-like creatures found in aquatic habitats (Zamani *et al.*, 2013; Kalasariya *et al.*, 2021). Yu *et al.*, (2014) report that they can range in size from tiny to 60 meters long. Seaweeds resembleplants, but lack a specific vascular system (Yu *et al.*, 2014; Kalasariya *et al.*, 2021; Morais *et al.*, 2021). This vascular system is an internal conducting system that links all organs and distributes fluids, nutrients, and various signalling molecules throughout the plant body (Lucas *et al.*, 2013). Seaweeds acquire nutrients directly from saltwater, eliminating the need for additional nutrients. Photosynthetic organisms use sunlight to produce ingredients for growth (Sudhakar *et al.*, 2018). Seaweeds contain chlorophyll, a pigment that helps absorb sunlight for photosynthesis and gives them their green color (Chen *et al.*, 2017). Seaweeds' unique hues, including red and brown, are due to other pigments contained in them (Morais *et al.*, 2021). Some seaweeds utilize a rootlike structure called holdfasts to anchor themselves to the water floor. Floating seaweeds use air sacs to stay above the water's surface and capture sunlight for photosynthesis. Stipe, a stem-like structure, anchors seaweed blades to the water's surface. Blades are the main photosynthetic leaflike flattening portions that absorb sunlight. Thallus is a seaweed's body-like structure capable of photosynthesis (Sudhakar *et al.*, 2018).

Seaweed-Based Bioactive Components

Seaweeds are known for producing a wide variety of bioactive macromolecules. Seaweeds include many components such as colour, phenolic compounds, lipids, proteins, vitamins, minerals, and polysaccharides (Bedoux *et al.*, 2014). Algae are a major source of bioactive compounds, including sulfated and non-sulfated polysaccharides (Jiménez-Escrig *et al.*, 2011; Jesumani *et al.*, 2019; Venugopal, 2019; Hentati *et al.*, 2020; Rengasamy *et al.*, 2020). Polysaccharides are classified as sulfated (agars, carrageenans, fucoidans, and galactans) or non-sulfated (alginates, laminaran) (Rupérez *et al.*, 2013; Hentati *et al.*, 2020). Seaweeds contain three types of pigments: chlorophyll, carotenoid, and phycobiliproteins. These pigments have antiangiogenic, anticancer, anti-inflammatory, antioxidant, and immunomodulatory properties. They can also be used as food dyes (Merilyn Que *et al.*, 2022). Chlorophylls, greenish pigments that are soluble in lipids, are crucial for photosynthesis in seaweeds. Algal carotenoids often consist of astaxanthin, carotenes, fucoxanthin, lutein, lycopene, neoxanthin, violaxanthin, and zeaxanthin. Carotenoids are terpenoid chemicals that support seaweed photosynthesis. Fucoxanthin, generated from brown seaweed, is the most common carotenoid and has potential applications in food.

Finally, phycobiliproteins are water-soluble pigments found in proteins. Phycobiliproteins include three pigments: phycoerythrin, the primary red pigment, allophycocyanin, the light-blue pigment, and phycocyanin, a blue pigment. The three pigments exhibit distinct protein, bilin, and spectrum characteristics (Aryee *et al.*, 2018; Cherry *et al.*, 2019).

2.2.1. Phenolic compounds

Seaweed includes a variety of phenolic compounds, including catechins, flavonoids, phenolic acids, phlorotannin's, and tannins. Seaweed species significantly influence the kind and quantity of phenolic chemical extractions. Green and red seaweeds have high levels of phenolic acids, flavonoids, and bromophenols. Brown seaweeds contain complex polymers, including phlorotannin's and phloroglucinol oligomers (1,3,5- trihydroxybenzene). Seaweed polyphenols have been associated to several biological activities, such as antibacterial, anticancer, antiinflammatory, anti-obesity, antioxidant, antiproliferative, antitumor, and antiviral properties (Montero *et al.*, 2017; Gómez- Guzmán *et al.*, 2018; Cota's *et al.*, 2021).

2.2.2 Proteins

Protein content in seaweed varies by species, seasonal cycle, and fluctuation causes. According to Fleurence *et al.*, (2018), red seaweeds account for up to 47% of dry weight, green seaweeds for 35%, and brown seaweeds for a lesser percentage. Seaweeds' protein content has been overestimated due to the presence of nonprotein nitrogen. Therefore, nitrogen-to-protein

conversion ratios below 6.25 are advised for feed components (Makkar *et al.*, 2016). Seaweeds include important amino acids such as glycine, alanine, proline, arginine, glutamic acid, and aspartic acid (Gullón *et al.*, 2020). Phycobiliproteins are a kind of algal protein that can create peptides with antihypertensive properties by inhibiting angiotensin through enzymatic breakdown.

2.2.3 Lipids

Seaweeds typically contain modest lipid contents of up to 5% of their dry weight (Misurcova *et al.*, 2011). Some species have a total lipid content above 10% dry weight, making them suitable for oil-based products (Gosch *et al.*, 2012). Total lipid concentration fluctuates according to geographical location, interactions, light intensity, salinity, seasonal changes, species, and temperature (Susanto *et al.*, 2019). Seaweed lipids include high levels of Polyunsaturated Fatty Acids (PUFAs), including Linolenic acid, Stearidonic acid, Eicosapentaenoic acid (n-3 PUFA), and Arachidonic acid (n-6 PUFA). Lipids include bioactive compounds, such as sterols (Luo *et al.*, 2015; Pérez *et al.*, 2016; Susanto *et al.*, 2019). Sterols, primarily cholesterol and clionasterol, have numerous nutritional and biological benefits, including anticancer, antiobesity, antioxidant, antitumor, and antiviral properties. They also help prevent cardiovascular disorders. According to Kendel *et al.*, (2015), fecosterol and isofucosterol are the primary nutritious components found in seaweeds.

2.2.3. Vitamins and minerals

Seaweeds have a high concentration of minerals (8-40%), including Na, K, Mg, Fe, and others (Cofrades *et al.*, 2017; Lorenzo *et al.*, 2017). Calcium is the most visible mineral and has the highest concentration in plant sources. They have a lower Na/K ratio than other foods in Western diets, which promotes cardiovascular health (Circuncisão *et al.*, 2018). Consuming seaweeds can help alleviate iodine shortage due to their high iodine content.

Seaweeds are rich in fat-soluble vitamins like A, D, E, and provitamin A, as well as watersoluble vitamins like C, folic acid, pantothenic acid, niacin, riboflavin, and vitamins B12, B6, B3, B2, and B1 (Hentati *et al.*, 2020). The vitamin content in seaweeds varies by species, resulting in relatively low levels (Škrovánková *et al.*, 2011). The vitamin E concentrations in green seaweeds ranged from 8.8-12.0 mg/kg, red seaweeds from 10-26 mg/kg, and brown seaweeds from 1.6-122 mg/kg dry weight. (Bianca Rosa *et al.*, 2018). Green seaweeds contain 0.0347-1.25g/100g of vitamin C, red seaweeds 0.0353-1.61g/100g, and brown seaweeds 0.0345-1.85g/100g dried weight. For essential vitamin B3, green

seaweeds range from 0.005-1.0g/100g, red seaweeds from 0.0951-0.10g/100g, and brown seaweeds from

0.612-0.90g/100g dried weight (Hentati *et al.*, 2018). In addition, the levels of Vitamin B12 in seaweeds vary. Green seaweeds vary from 0.06 to 0.786 g/100 g, red seaweeds from 0.0961 to 1.34 g/100 g, and brown seaweeds from 0.0164 to 0.0431g/100 g dry weight (Hentati *et al.*, 2018; Cherry *et al.*, 2019).

2.2.4 Polysaccharides

Phycocolloids are seaweed-derived hydrocolloids that generate viscous solutions when combined with water. In 2019, the expected worldwide import and export of seaweeds and seaweed-based hydrocolloids were \$2.9 billion and \$2.65 billion, respectively (FAO, 2021b). Polysaccharides produced from phycocolloids, such as alginate, agar, and carrageenan, have commercial value in the pharmaceutical and nutraceutical industries (Gnanavel *et al.*, 2019). Seaweed polysaccharides vary per species, ranging from 4% to 76% by dry weight. Brown seaweeds include alginates, *Gelidium*, *Pterocladia*, *Gelidium*, and *Gracilaria* fucoidans, and laminarin, whereas red seaweeds contain carrageenan's and agarin's.

Green seaweeds contain ulvans. Seaweed polysaccharides include both sulphated (agars, carrageenan's, fucoidans, and galactans) and non-sulphated (alginates, laminarin) polysaccharides with high levels of dietary fibre. Prebiotic characteristics have been linked to antibacterial, anticancer, anticoagulant, antihyperlipidemic, anti-inflammatory, antiobesity, antioxidant, antiviral, gastroprotective, and immunomodulatory activities (Seong *et al.*, 2019; Gullón *et al.*, 2020; Hentati *et al.*, 2020). Hydrocolloids, including agar, carrageenan, and alginates, are also known as phytochemicals. They are used in several sectors, including food, dairy, confectionery,

textiles, paper, and manure in some nations. (Pal *et al.*, 2014). Red seaweeds (Rhodophyta) like contain agar, which is made up of two polysaccharides: agarose (for gelling) and agarpectin. However, agarose accounts for around 70% of the mixture. Agar includes hydrophilic galactans such as $\alpha(1-4)$ -3,6-anhydroL-galactose and $\beta(1-3)$ -D-galactose (Lee *et al.*, 2017). It is the first hydrocolloid with the European registration number E406. The FDA has recognized agar as Generally Recognized as Safe (GRAS) for use as a food additive, with around 80% of it being manufactured globally. The remaining 10-20% worked in the pharmaceutical and biotechnology sectors. Agar may be utilized in several ways according on its quality. Low-grade agar is used in culinary and industrial applications, including adhesives, casting, impressions, paper coating,

textile printing, dyeing, and more. Medium grade agar is used as a gel substrate in several medical and pharmaceutical applications, including biological culture media, anticoagulants, bulking agents, capsules, laxatives, and tablets. Highly purified agar is a highgrade agar used in intermolecular biology separation methods such as electrophoresis, gel chromatography, and immunodiffusion (Pal *et al.*, 2014; Abdul Khalil *et al.*, 2018). Carrageenan (European registration name E407) is derived from red seaweeds, specifically *Kappaphycus alvarezii*, *E. denticulata*, *E. spinosum*, *B. gelatinae*, *C. crispus*, *Gigartina sp.*, and *Hypnea sp.*, accounting for up to half of the dry weight (Abdul Khalil *et al.*, 2018). A category of naturally occurring water-soluble sulfated galactans having alternating backbones of $\alpha(1-4)$ -3,6-anhydro-Dgalactose and $\beta(1-3)$ - D- galactose(.Subaryono, 2018).Carrageenan is categorized into three kinds: iota (ι)- carrageenan, kappa (κ)- carrageenan, and lambda (λ)carrageenan. Other types, such as μ -carrageenan and ν carrageenan, have also been identified (Rhein-Knudsen *et al.*, 2017). Carrageenan is often used in many foods as an emulsifier, thickener, and stabilizer. Carrageenan is often used in meals as an emulsifier, thickening, stabilizer, and protective covering for fresh-cut packaged goods. Carrageenan, a phycocolloids found in red seaweeds, is tough to define due to its diverse species and composition. (Abdul Khalil *et al.*,2018). Alginates are generated from brown seaweeds such *Ascophyllum nodosum*, *E. maxima*, *L. digitata*, *L. hyperborean*, *L. japonica*, *L. nigrescens*, *M. pyrifera*, and *Sargassum sp.* Alginates are polymers composed of $\alpha(1-4)$ connected α Galacturonic and β -D-mannuronic acids (Ramnani *et al.*, 2012; Abdul Khalil *et al.*, 2018). It is mostly used in the food and pharmaceutical sectors for binding metal ions and creating viscous solutions. It is used as a gelling and sizing agent for cotton yarn in the textile industry. Alginate has two types: acid and salt. Asalginic acid is a linear polyureic acid, whereas salt is a significant component in brown seaweed cell walls, accounting for 40-47% of algal biomass.

2.3. Seaweed Extraction

Modern techniques, including Enzyme Assisted Extraction (EAE), Microwav Assisted Extraction (MAE), Ultrasound-Assisted Extraction (UAE), Supercritical Fluid Extraction (SFE), and Pressure Solvent Extraction (PSE), have been used to extract novel metabolites from seaweed without degradation (Cikos *et al.*, 2018).

2.4. Soxhlet extraction method

Soxhlet and other solvent-based extraction procedures are commonly used to extract these chemicals from matrices. Although this approach has several advantages— samples can have fresh solvent contact throughout the operation, no filtering is required, simple handling, etc. Soxhlet extraction is sluggish and requires many organic solvents, which must then be removed. A supercritical fluid possesses temperatures and pressures that exceed its critical limits (SF). While SFs have better diffusion and lower viscosities, their densities and dissolving properties are comparable to other liquids (Kadam *et al.*, 2015). When the supercritical state is reached, the solute mass transfer from the matrix to the solvent is facilitated because the supercritical fluid density is equivalent to a liquid and the viscosity is low in the zone between liquid and gas. (Matos *et al.*, 2021). CO₂ is the most often used solvent because its critical point allows for the extraction of labile compounds. Other probable supercritical solvents include acetone, methanol, ethanol, methane, ethane, propane, ethylene, and propylene. (Kadam *et al.*, 2015; Abdul Khalil *et al.*, 2018).

2.5. Anti-Oxidant property of seaweed

Seaweeds include different antioxidants, including polysaccharides, dietary fibres, minerals, proteins, amino acids, vitamins, polyphenols, and carotenoids (Burtint *et al.*, 2003).

Lesser *et al.*, (2006) found that seaweed generates antioxidants to protect against environmental stress. Seaweed may include new antioxidants. Natural antioxidants are preferred over synthetic antioxidants due to their lack of chemical pollutants and several benefits. Natural antioxidants are safe for use in medication, nutritional supplements, nutraceuticals, and cosmetics to improve consumer health, reduce illnesses, and enhance immune system performance (Shahidi, 2009). The anti-oxidant activity of seaweed can be measured using Radical DPPH (2,2-Diphenyl-1-picrylhydrazyl).

In this present study, objectives are postulated to In Vitro propagation extraction and characterisation of secondary metabolites from *Gracilaria* spp. Rhodophyceae macroalgae and evaluated for its anti-proliferation and anti-oxidant activity.

CHAPTER 3

AIM:

To acquire a substantial quantity of seaweed culture through in vitro propagation

OBJECTIVE:

- To collect and identify the seaweed from, South east coast of India. Viz Pattukottai, Tamil Nadu
- To perform in Vitro propagules for callus development.
- To extract phytochemical from solvent extraction method.
- To characterize and purify the extraction by TLC, column chromatography.
- To extract the antiproliferation and antimicrobial properties of the solvent
Phytochemical.

CHAPTER 4

MATERIALS AND METHODS

4.1 Sample collection



Fig.4.1 Seaweeds collected from Pattukottai

Seaweed sample is collected from Pattukottaieast coast of India. The genus *Gracilaria* is widespread in tropical warm water. Six of these species, *Gracilariacrassa*, *Gracilaria corticata*, *Gracilaria dura*, *Gracilaria edulis*, *Gracilaria fergusonii*, and *Gracilaria foliifera*, have the capacity to produce agar.

Preparation of MS Medium

Stock solution was prepared using macro and micro nutrients, minor nutrients, vitamins and amino acids, and potassium iodine. All the stock was prepared for 250ml and pour together made volume up to 1 litre by adding water. The stock was filtered using membrane filter paper.

Table 1.1: Stock Preparation

MACRONUTRIENT (10)	1L	250ML
NH ₄ NO ₃	16.5g	4.13g
KNO ₃	19.00g	4.25g
MgSO ₄ .7H ₂ O	3.70g	0.925g
CaCl ₂ .2H ₂ O	4.40g	1.10g

MINOR NUTRIENT (100X)	1L	250ML
MnSO ₄ .4H ₂ O	2.23g	0.56g
ZnSO ₄ .4H ₂ O	0.86g	0.21g
H ₃ BO ₃	0.62g	0.51g

MICRONUTRIENT (100X)	1L	100ML
NaMoO ₄ .2H ₂ O	2.5mg	2.5mg
CuSO ₄ .5H ₂ O	0.25mg	0.25mg
CoCl ₂ .6H ₂ O	2.5mg	0.25mg

KI	1L	250ML
KI	0.83g	0.0207g

VITAMINS FOR AMINO ACIDS (100X)	1L	10ML
NICOTONIC ACID	50mg	5mg
PYRODOXIN	50mg	5mg
THIANINE HCL	10mg	1mg
GLYCINE	200mg	20mg

4.1.1. MS MEDIA PREPARATION FROM STOCK SOLUTION

Eight boiling tubes were prepared, and 40 ml of MS medium stock solution was measured out. The pH was adjusted to 6.5. Subsequently, 5 ml of MS medium was dispensed into each boiling

tube. Three percent sucrose and 0.5% agar were then added, with 150 mg of sucrose and 40 mg of agar introduced into each boiling tube. An additional 5 ml of MS medium was poured into each boiling tube containing sucrose and agar. The tubes were autoclaved at 121°C for 30 minutes.

4.1.2. INOCULATION OF SEaweEDS IN MS MEDIUM

The Petri dishes, conical flasks, forceps, and scalpel underwent sterilization at 121°C for 30 minutes. Three conical flasks were then prepared. The first contained 50 ml of 70% ethanol, the second held 50 ml of 0.1% HgCl₂ (mercuric chloride), and the third was filled with 100 ml of deionized water. Next, the laminar flow hood was sterilized using 70% ethanol. Petri plates were placed under the laminar airflow with UV light present. Three of the plates were filled with deionized water, one with 70% ethanol, and one with HgCl₂(mercuric chloride), while the last one remained empty. The scalpel was sterilized, and the provided seaweed sample was cut into pieces measuring 1-2 cm. Each sample was sequentially dipped into the prepared petri plates: first into the deionized water, then into the HgCl₂ solution, and finally into the 70% ethanol.

Additionally, the samples were dipped into two more petri plates containing deionized water. Subsequently, the seaweed samples were transferred directly into test tubes, which were then cotton-plugged and set aside for culture. The seaweed was then incubated at room temperature under light conditions ranging from 50 to 150 $\mu\text{mol}/\text{m}/\text{sec}$ for a week. Regular monitoring of the seaweed culture within the incubator was conducted, with eventual harvesting of the culture for subsequent Extraction of the component and Analysis.

4.2. EXTRACTION OF SECONDARY METABOLITES FROM SEaweED

4.2.1 SOXHLET EXTRACTION METHOD

Solid material is placed in a thimble, and the Soxhlet apparatus was then positioned onto a flask containing the extraction solvent. The Soxhlet was heated to reflux. As a result, the solvent vapor travels up to the distillation arm and floods into the chamber housing the thimble filled with solid material. The solid slowly fills with the warm solvent, and the desired compound dissolves in it. When the Soxhlet chamber is nearly full, the solvent is

emptied from the chamber using a siphon, allowing it to run back down to the distillation flask. This cycle can be repeated up to 10 times. During each cycle, a portion of the compound dissolves in the solvent. After several cycles, the desired compound becomes concentrated in the distillation flask. After extraction, the solvent was typically removed using a rotary evaporator, resulting in the extracted compound. The non-soluble portion of the extracted solid remains in the thimble and was usually discarded.

4.3. PURIFICATION AND SCREENING

4.3.1. COLUMN CHROMATOGRAPHY

Column chromatography stands as one of the most prevalent techniques for protein purification. It operates on the principle wherein molecules from a mixture are applied onto a solid surface, and the stationary phase, alongside a mobile phase, facilitates their separation as they move. In this process, a column was prepared utilizing silica gel as the stationary phase. The mobile phase, consisting of ethanol and water in a 10:3 ratio, is then allowed to flow through the column. Subsequently, the solvent obtained from the Soxhlet extraction method was introduced into the column and subjected to purification.

4.3.2. THIN LAYER CHROMATOGRAPHY

The solvent, purified through column chromatography, was applied to the silica-coated plate. A mobile phase was then prepared with ethyl ether and acetone in a 1:3 ratio. This solvent mixture was allowed to migrate across the silica gel plates for 20 minutes. The separated components are measured using a measuring table to confirm the presence of squalene. The R_f value was determined using the following formula.

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

4.4. ANTIOXIDANT ACTIVITY OF SECONDARY METABOLITES

4.4.1. DPPH ANTIOXIDANT ASSAY

ACETATE BUFFER

Acetate buffer was prepared by combining 0.820g of sodium acetate with 99.4ml of distilled water, followed by the addition of 0.6ml of acetic acid. The pH of the solution is adjusted to

5.5.

METHANOLIC BUFFER

4ml of acetate buffer was added with 6ml of methanol.

STANDARD SOLUTION

Mix 10 mg of ascorbic acid with 1ml of methanolic buffer. DPPH

STANDARD

20 μ l of ascorbic acid is introduced into an Eppendorf tube, followed by the addition of 2.98ml of methanolic buffer. Similarly, 40 μ l, 60 μ l, 80 μ l, 100 μ l, and 120 μ l of ascorbic acid are each combined with methanolic buffer in separate Eppendorf tubes, with volumes of 2.96ml, 2.94ml, 2.92ml, 2.92ml, and 2.90ml respectively.

Next, 3.94mg of DPPH was dissolved in 100ml of ethanol. Subsequently, 50 μ l of the solution from each Eppendorf tube is transferred to individual test tubes. To each test tube, 2.96ml of DPPH solution is added, and the mixtures are incubated for 30 minutes.

Following incubation, 200 μ l from each test tube is transferred to a well plate and the absorbance is measured at 570nm.

4.4.2. PHENOLIC ESTIMATION

GALLIC ACID STANDARD

A methanol stock solution was prepared by mixing 10mg of gallic acid with 2ml of methanol. From this stock solution, 50 μ l is extracted using a micropipette and transferred to a tube, followed by the addition of 950 μ l of methanol. Separately, a solution of 7g of Na₃CO₃ in 100ml of distilled water is prepared. Folin reagent is then combined with distilled water in a 1:1 ratio to create a solution. From the gallic acid stock solution, aliquots of 100 μ l, 150 μ l, 200 μ l, 250 μ l, 300 μ l, and 350 μ l are extracted and each combined with methanol to a final volume of 1ml.

Subsequently, 50 μ l from each tube is transferred to individual test tubes. To each test tube, 50 μ l of Folin reagent is added, and the mixtures are incubated for 5 minutes. Following this, 9ml of Na₂CO₃ is added to each tube and the mixtures are incubated for 30 minutes. After incubation,

200µl from each tube is transferred to a well plate, and the absorbance is measured at 760nm.

4.4.3. MTT ASSAY FOR ANTI-PROLIFERATION ACTIVITY

Cell line and culture:

MCF 7 Cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO₂ at 37°C.

In Vitro assay for anti-cancer activity: (MTT assay) (Mosmann, 1983)

Cells (1×10^5 /well) were plated in 24-well plates and incubated in 37°C with 5% CO₂ condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC₅₀) was determined graphically. The % cell viability was calculated using the following formula:

$$\% \text{ Cell viability} = \text{A570 of treated cells} / \text{A570 of control cells} \times 100$$

Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

CHAPTER 5 RESULTS AND DISCUSSIONS

5.1. Sample collection



Fig 5.1. Sample collection

Seaweed obtained from Pattukottai east coast of India. The genus *Gracilaria* is common in tropical warm seas (McLachlan and Bird 1986). There are around 180 species of *Gracilaria* in the world, 32 of which have been documented from India. According to Krishnamurthy (1991), six species (*Gracilariacrassa*, *Gracilaria corticata*, *Gracilaria dura*, *Gracilaria edulis*, *Gracilaria fergusonii*, and *Gracilaria foliifera*) may make agar. Aside from *G. dura*, additional taxa may be found in abundance along India's southeast coast. Wild seaweed's share of global seaweed output fell from 28% in 1980 to 4.5% in 2010 (Cai et al.,2013) owing to overexploitation.

In conventional cultivation seaweeds were cultivated at varied depths, using bamboo rafts were sown with 2 kg seed material (100 propagules m⁻²) and placed on the surface of the water (0-m depth) and submerge (below 1-m depth). Harvested after 45 days. But the invitro propagation methods helps in producing biomass in high pure form within closed system at laboratory conditions.

5.2 Sample inoculation



Fig5.2. Seaweed inoculation in MS media

MS media is prepared using macro, micro nutrients, vitamins, and irons. Seaweed was inoculated on the MS media and kept for incubation at room temperature with a light intensity ranging from 50 to 150 $\mu\text{mol}/\text{m}^2/\text{sec}$.

5.3. Seaweed extraction



Fig:5.3. seaweed extraction

Soxhlet extraction methods were widely utilized for extracting compounds from matrices. Although this strategy has various advantages, such as the ability to maintain fresh solvent contact throughout the operation, the absence of the need for filtration, the ease of handling, and so on. Soxhlet extraction is slow and needs many organic solvents, which must be eliminated. This technique is used to extract a seaweed sample with benzene as the solvent.

5.3. Purification and screening

5.3.1 Column chromatography



Fig: 5.4. Column Chromatography (Gracilaria)

Chromatography is a process that separates the constituents of a mixture based on their interactions with two phases known as a mobile phase and a stationary phase. The mobile phase comprises of sample components that travel through the system. The stationary phase is retained in situ with some support. The quantity of molecule or chemical eluting from the column as a function of elution time or the volume of mobile phase passing through the apparatus. The resulting plot of reaction vs. time or volume is known as a chromatogram. The chromatogram shows several peaks representing distinct sample components being eluted from the column. The chromatogram shows several peaks representing distinct sample components being eluted from the column. The retention time or volume of each peak may be used to identify the chemical, whilst the area or height of the peak can be used to calculate the quantity of compound present.

5.3.2. Thin layer chromatography

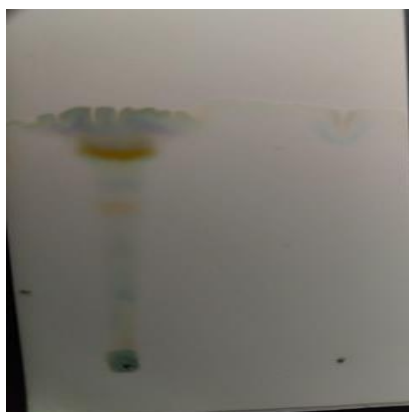


Fig:5.5. Thin layer chromatography (Gracilaria)

The samples were spotted on the stationary Silica gel G adsorbent on the plate and put in an enclosed environment to expand in the presence of mobile phase. Typically, a solvent or mixture of solvents is utilized as the mobile phase. The sample travels over the plate according to the type of analytes present. Analytes are identified based on their migration distance across the stationary phase and capacity to absorb UV light or react with chromogenic derivatization spray reagents (Wilson *et al.*, 2014). TLC-based detection of Gracilaria seaweed compounds, seen under visible light (a), bio-autographic screening of active bioactive compounds (b), active compound identification (c), and active compound purification using preparative TLC (d). Rajauria and AbuGhannam (2013) studied the biological features of Gracilaria seaweed's active component, including its chromatographic profile, R_f value, colour, bioactivity, antioxidant capacity, and spectrum.

5.4. ANTI-OXIDANT ACTIVITY

5.4.1. DPPH Assay Standard Ascorbic Acid

Calculation of % Radical Scavenging and IC 50 DPPH assay				
Concentration	Control	Sample	% RSA	IC50
50	0.08	0.102	27.5	5.256225
100	0.08	0.107	33.75	13.37746
150	0.08	0.112	40	21.49869

200	0.08	0.119	48.75	29.61993
250	0.08	0.124	55	37.74116
300	0.08	0.13	62.5	45.86239

Table:5.2. DPPH Assay of Secondary metabolites

Calculation of % Radical Scavenging and IC 50 DPPH assay				
Concentration	Control	Sample	% RSA Squalene	IC50
50	0.163	0.222	36.1962	5.256
100	0.163	0.247	51.5333	13.377
150	0.163	0.262	60.7361	21.498
200	0.163	0.28	71.7791	29.619
250	0.163	0.297	82.2085	37.741
300	0.163	0.31	90.1840	45.862

Ascorbic acid is combined with methanolic buffer in Eppendorf tubes, followed by 3.94mg of DPPH in 100ml of ethanol. The mixtures are incubated for 30 minutes, then 200µl from each tube is transferred to a well plate and absorbance is measured at 570nm

5.4.2. Phenolic Content Standard Gallic Acid

Table:5.3. Phenolic Content Standard Gallic Acid

Gallic Acid standard		
Concentration	Absorbance	Gallic Acid $\mu\text{g/mL}$
0.1	1.075	0.0814
0.5	1.451	0.4574
1	2.487	1.4934
2.5	2.957	1.9634
5	3.69	2.6964

Gallic Acid Standard		
Concentration	Absorbance	mg/g
50	0.3514	35.8136
100	1.451	145.7736
150	2.487	249.3736
200	2.957	296.3736
250	3.69	369.6736

Expressed as mg gallic acid/ 100mg of extracted substance $(n - 1)x^2$
Absorbance x Slope

$$R^2=.09832$$

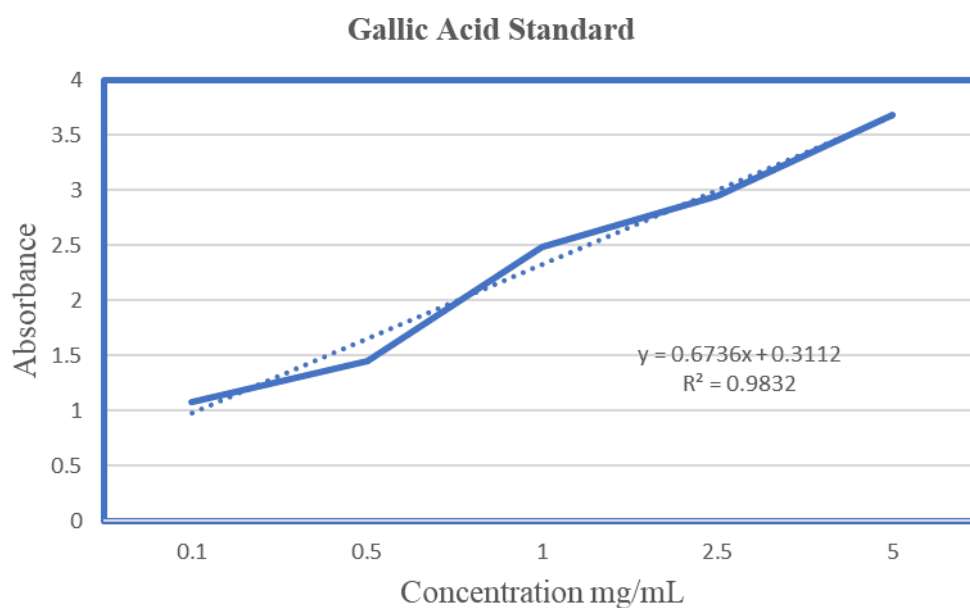


Fig :5.6. Gallic Acid Standard

A methanol stock solution is prepared by mixing gallic acid with methanol. 50 μ l is extracted and added to a tube. Na₃CO₃ is prepared in distilled water and Folin reagent is combined. Aliquots are extracted and combined with methanol. Test tubes are added with Folin reagent and Na₂CO₃ and incubated for 5 minutes. Absorbance is measured at 760nm.

Table:5.4: Antiproliferation effect of Sample SA on MCF-7 cell line

S.No	Cell viability (%)	Concentration (μ g/ml)	Absorbance (O.D)
1	47.83	1000	0.487
2	53.63	500	0.546
3	59.03	250	0.601
4	64.14	125	0.653

5..4.Anti proliferation effect of Sample SA on MCF-7 cell line

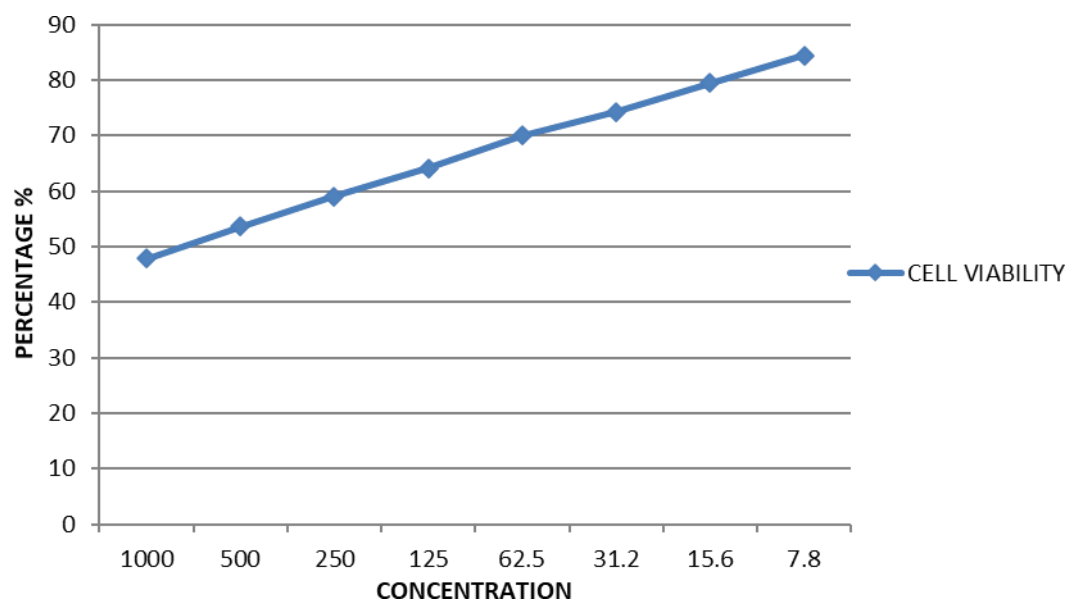
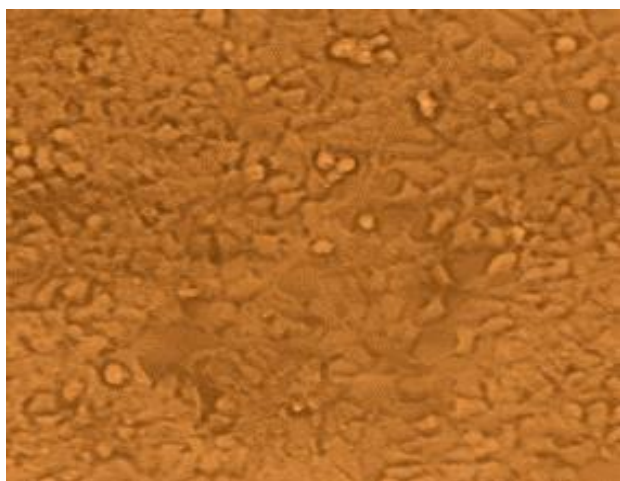


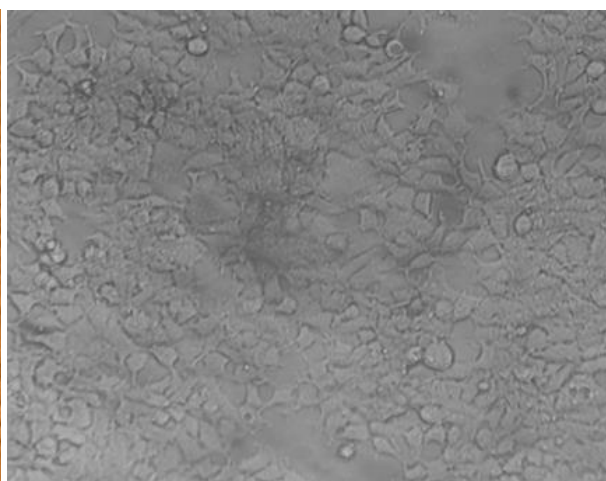
Fig :5.6. Anticancer effect of Sample SA on MCF-7 cell line

Table5.5. Anticancer effect of Sample SA on MCF-7 cell line

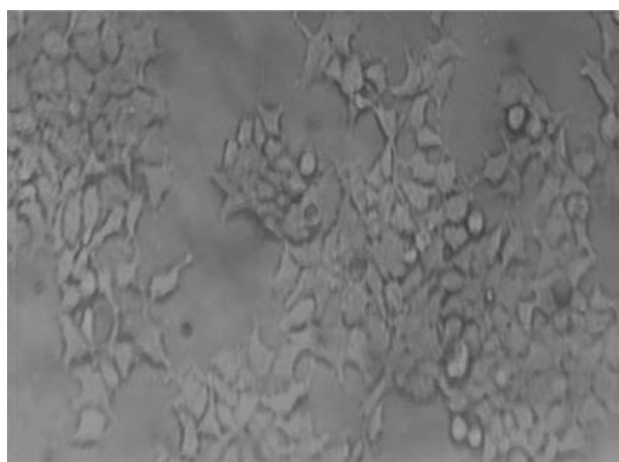
69.94	5	Cell control	0.712
74.26	6	62.5	0.756
79.46	7	31.2	0.809
84.67	8	15.6	0.862
100	9	7.8	1.018



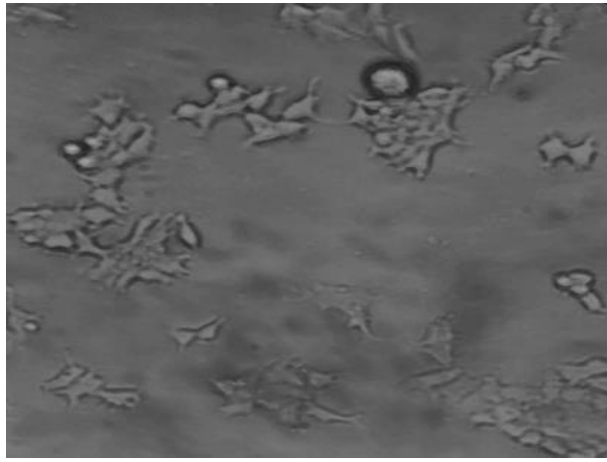
Control Cell



7.8 µg/mL



500 µg/mL



1000 µg/mL

Fig:5.7: Anticancer effect of Sample SA on MCF-7 cell line

The MTT test was used to assess the cytotoxic effect of seaweed extract (*Gracilaria* sp) against MCF-7 cancer cell lines. The antitumor efficacy of seaweed extract at different doses against MCF-cell lines was demonstrated. Increasing the concentration of seaweed extract to 100, 150, 200, 250, and 300 µg/ml resulted in lower cell viability. *Gracilaria* sp. extract showed cytotoxic effects against MCF-7 cancer cell lines at concentrations ranging from 100 µg/ml to 300 µg/ml. The reduction in cell density indicates cell death. The extract treated OD value of *Gracilaria* was used to compute the IC₅₀ value, which ranged from 200 µg/ml to 250 µg/ml for MCF-7 cancer cells. Cell lines that show promising cytotoxic potential.

CHAPTER 6

SUMMARY AND CONCLUSION

Secondary metabolites were extracted from seaweed samples cultivated using in vitro propagation techniques and seaweed biomass from wild holds significant promise in various therapeutic applications. The characteristics inherent in the extracted compounds play a pivotal role in determining their capacity to generate desired therapeutic properties and specific physiology effects. Particularly noteworthy is the phenolic-rich composition found in the propagated seaweed, which, when combined with other constituents, demonstrates considerable potential for providing beneficial effects. Studies suggest that these compounds may exhibit promising benefits, particularly in the context of breast cell health and in the soothing of burns. Furthermore, in vitro studies have been conducted to assess the properties of squalene derived from seaweed. These studies serve to deepen our understanding of its therapeutic potential and elucidate its mechanisms of action.

MS media is prepared from stock solution. Inoculation of seaweed is done using MS medium. Then seaweed extraction is done using Soxhlet extraction method.

Subsequently, the solvent obtained from the Soxhlet extraction method is introduced into the column and subjected to purification. Column chromatography stands as one of the most prevalent techniques for protein purification.

Thin layer chromatography, a technique used to separate mixtures of compounds based on differences in polarity. The solvent purified through column chromatography, is applied to the silica coated plate. The separated components are measured using a measuring table to confirm the presence of squalene.

Furtherly, DPPH antioxidant assay, phenolic estimation, and MTT assay for anti- cancer activity are classified under the molecular characteristics. DPPH assay is a widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. The DPPH antioxidant assay was determined using DPPH standard which comprises acetate buffer, methanolic buffer and standard solution.

Phenolic estimation was done to evaluate total phenolic content of prepared plant extracts and its antioxidant properties. The total phenolic content was determined quantitatively using the Folin Ciocalteu reagent, with Gallic acid as the standard.

The MTT assay is a colorimetric method used to quantify cellular metabolic activity as an indicator of cell viability, proliferation, and indirect cytotoxicity. MCF-7 is a human breast cancer cell line with oestrogen, progesterone, and glucocorticoid receptors.

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