RESEARCH
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THE INTERACTION OF BACTERIAL BIOFILM AND SEAWEED EXTRACT AGAINST PATHOGEN *Proteus mirabilis*

Running title: THE INTERACTION OF BACTERIAL BIOFILM AND SEAWEED EXTRACT AGAINST PATHOGEN *Proteus mirabilis*
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Abstract
Introduction: Proteus mirabilis is a Gram negative bacterium that is a frequent cause of catheter-associated urinary tract infections (CAUTIs). Its ability to cause such infections is mostly related to the formation of biofilms on catheter surfaces. Nowadays, seaweeds are considered a novel source of bioactive compounds and produce a great variety of secondary metabolites exhibiting broad spectrum of biological activities. In this we consider the potentiality of seaweed - sargassum.
Aim & objectives: The present study aims to explore the antibacterial activity of sargassum seaweed extracts against Proteus mirabilis
Materials and methods: By examining its morphological description, existing literature references, and employing herbarium comparison studies, the Sargassum spp. identification was completed. Methanolic extract of sargassum was prepared. Antibacterial activity was studied using microscope and pcr analysis of virulence gene.
Results: The methanolic extract of sargassum has a maximum antibacterial activity of 68.2% at a concentration of 100 microgram/mL. The strongest peak in the FTIR analysis of Sargassum indicates that phenol is the main component in this seaweed extract.
Conclusion: In the present investigation methanolic extract of sargassum showed a promising antibacterial effect against the biofilm of Proteus mirabilis.
Keywords: Proteus mirabilis,Bacterial biofilm, Sargassum, Antibacterial activity.

INTRODUCTION:

Urinary tract infections (UTI) rank among the most prevalent infectious disorders in the world. Every year, UTI is diagnosed in over 150 million persons. An illness affecting the kidneys, ureters, bladder, or urethra is known as a urinary tract infection. Prior to being expelled from the body, urine passes through these structures. When Gram negative bacteria like Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis enter the urine and start to multiply, an infection practically always results. The

infection typically begins at the urethral opening, where urine exits the body and ascends into the urinary system.

These bacteria are normally found in the colon and the area around the anus. According to Kalaivani et al. (2016), the symptoms include a very high fever, shivering chills, nausea, and vomiting. Acute or chronic kidney infection (pyelonephritis), or even irreversible kidney damage, can develop from a UTI infection if it is ignored or improperly managed.

Rod-shaped Gram-negative bacteria from the Enterobacteriaceae family are included in the genus Proteus. They are widely distributed throughout the ecosystem, primarily in the water, soil, and gastrointestinal systems of people and animals. Proteus species make up less than 0.05 percent of the human gut microbiota in healthy individuals. Proteus mirabilis is the Proteus species that infects people the most frequently overall. It is an opportunistic pathogen that is linked to a number of human disorders, including those affecting the skin, eyes, ears, digestive system, and respiratory system. According to Jamil et al. (2020), P. mirabilis is also a frequent cause of complex urinary tract infections (UTIs) in patients with structural or functional issues. This can lead to catheter-associated urinary tract infections (CAUTIs), which is particularly problematic for individuals with long-term indwelling urinary catheterization. P. mirabilis has the unusual capacity to produce crystalline biofilms, which eventually cause catheters to become encrusted and obstructed. Patients may subsequently have painful bladder distension, reflux, and pyelonephritis along with urine retention. Then, fatal consequences including septicemia and endotoxic shock may be on the horizon. Additionally, the removal of the catheter may result in injury to the bladder mucosa and urethra. Replacement of clogged catheters may also necessitate more nursing visits and emergency referrals. Additionally, P. mirabilis persistence in the urinary system has been connected to the crystalline biofilms' resistance to drugs and the human immune response.

P. mirabilis colonization of the intestinal tract serves as a reservoir for sporadic colonization of the periurethral area. Urinary catheters are infected with the bacterium that later spreads to the bladder during insertion. Exopolysaccharide synthesis and biofilm development are induced by adhesion to catheter surface or bladder epithelium. The highly-organized structures of single- or multi-species microbial communities known as biofilms are formed when microbial cells are permanently bonded to a surface as well as to one another. According to Flemming and Wingender (2010), cells in biofilms are immersed in a matrix that self-assembles from extracellular polymeric components like polysaccharides, proteins, lipids, and extracellular DNA. A multistage process, the creation of biofilms begins with reversible bacterial adherence to biotic or abiotic surfaces and progresses through irreversible attachment, microcolony formation, and mature biofilm development. In order for free bacteria to spread to other favorable environmental circumstances, the surface of the mature biofilm begins to shed them. Some bacteria species use biofilm development to survive in hostile environments and improve their resistance to drugs and the human immune system. According to Hoiby et al. (2010), biofilm-associated bacteria have 10-1,000 times more antimicrobial resistance than their planktonic counterparts.(1)

Some of the numerous virulence factors used by P. mirabilis to cause catheter-associated urinary tract infections (CAUTIs), including swarming motility, fimbriae, urease production, capsule polysaccharide, and efflux pumps, have been linked to their capacity to form biofilms. P. mirabilis is renowned for its remarkable swarming ability in particular and organism development on solid surfaces. These virulence factor causes the small, rod-shaped "swimmer cells" to differentiate into long, hyper-flagellated "swarmer cells," which can organize themselves into multicellular rafts. These cell rafts can move swiftly and cooperatively over solid surfaces. Therefore, the swarming mobility may make it easier for P. mirabilis to migrate along the catheter surface into the urine bladder, causing CAUTIs . Failure of P. mirabilis to migrate through catheter surfaces has been associated with loss of the swarming capacity caused by mutations. Additionally, swarmer cells frequently exhibit greater virulence factor expression, which improves their capacity to cling to catheter surfaces and bladder epithelium. Fimbriae (adhesins), which are attached to the protein coat created by bodily fluids on catheter surfaces or the catheter material itself (Downer et al., 2019), are the first step in the creation of biofilms on catheter surfaces. By whole genome sequencing, the genome of a P. mirabilis isolate contained at least 17 fimbrial operons. This is evidently represented by its strongest ability to cling to catheter surfaces among Gram-negative bacteria (Roberts et al., 2017), which is generally the highest among all sequenced bacterial genomes (Pearson et al., 2018; Scavone et al., 2016). Mannose-resistant/Proteus-like (MR/P) fimbriae are the most thoroughly researched fimbriae.(5)(2)

According to previous studies , MR/P fimbriae and PMF both have a significant impact on how P. mirabilis adheres to the bladder epithelium. According to a study by Jansen et al. (2018), MR/P fimbriae are neither required nor sufficient to start the production of a biofilm. Their constitutive expression, according to the same study, improved biofilm development. The critical function of MR/P in P. mirabilis' biofilm generation was demonstrated by the fact that MR/P mutants' biofilms were smaller than those produced by wild-type strains (Scavone et al., 2016). On the other hand, isogenic mutants unable to express PMF showed higher biofilm development, according to the same study. The capacity of P. mirabilis to create peculiar crystalline biofilms, which typically result in catheter encrustation and obstruction, is one of the organism's distinguishing characteristics. This complicates CAUTIs. The urease enzyme and capsule polysaccharides (CPSs) are two virulence factors that are known to play a part in the development of P. mirabilis crystalline biofilms. Almost all P. mirabilis clinical strains produce an incredibly powerful urease enzyme. The enzyme raises the local urinary pH by catalyzing the breakdown of urea in urine into ammonia. This is typically accompanied by local supersaturation, precipitation, and the formation of struvite crystals (ammonium magnesium phosphate) and hydroxyapatite crystals (calcium phosphate), which are both minerals that are typically found in urine. Ureolytic biomineralization is the mechanism by which such crystals are absorbed into the growing biofilm (Jacobsen and Shirtliff, 2011). The urease enzyme produces a lot of extremely alkaline ammonia, which is directly harmful to mammalian cells and can lead to tissue damage in addition to playing a significant part in the production of crystalline biofilms. (3)

Numerous antibiotic classes were categorized as common therapies for certain disorders. However, the incorrect and unchecked use of numerous antibiotics led to the development of antimicrobial drug resistance, which spurred the search for new treatment options and became a significant global health issue. According to Harnedy and FitzGerald (2018), macroalgae are a variety of marine organisms that have evolved to survive in a competitive marine environment. These marine organisms

are therefore acknowledged as potential producers of bioactive secondary metabolites, with many of these have been shown to exhibit intriguing biological activities. Many metabolites isolated from marine algae have been demonstrated to possess bioactive properties, and among the various compounds with functional properties, antioxidants are the most extensively studied. The importance of antioxidants in human health has also been demonstrated, increasing the interest in such products and their demand by consumers. Prior research has shown that prebiotics from algae, such as polysaccharides, phlorotannins, minerals, and omega-3 fatty acids, may also have antibacterial, antioxidant, and immunomodulatory effects that are beneficial to users. Marine algae serve as important resources for a variety of industries.(4)

According to Al-Saif et al. (2013) and Salem et al. (2011), marine algae have large concentrations of bioactive chemicals that are commonly employed in pharmaceuticals. Alkaloids, polyketides, cyclic peptides, polysaccharides, phlorotannins, diterpenoids, sterols, quinines, lipids, and glycerol were among the bioactive compounds recovered from marine algae. Consumers have recently become concerned about chemical preservatives in food. In addition to having health benefits, seaweed extracts can be utilized as a natural food preservative. A form of aquatic plant known as seaweed is found primarily in marine environments. Frequently referred to as seaweeds, they can be classified as red (Rhodophyta), brown (Phaeophyta), and green seaweeds (Chlorophyta) based on their pigmentation (Dawczynski et al., 2017). Algae are quickly growing organisms with significant mineral, carbohydrate, and protein compositions. The majority of them are carbohydrates in the form of cellulose and starch, making up 60–70% of the total algal composition. (4,5)

A recent study (Salem et al., 2011) showed that seaweed extracts in organic solvents have antibacterial properties. Active substances are present in gracilaria species. According to Kulik (2018), the antibacterial activity of the G. corticata extract is quite effective against the bacterium Proteus mirabilis. According to one publication, proximate composition analysis was used to estimate the nutritional value of sargassum meals. Seaweed meals had crude protein and fat contents of 10.28% and 0.418%, respectively. Following carbohydrates, which made up 46.61% of the seaweed, ash came in second with 32.46%. 10.25% of the sample contains crude fiber (Serrano Jr et al., 2015). (6)(7)

Many Asian countries have used seaweed as food since ancient times, and they commonly employ it as a source of biochemicals for use in food, medicine, and cosmetics. Sargassum is abundant in dietary fiber, brown algal starch, minerals, vitamins, excellent high-unsaturated fatty acids, and a healthy balance of key amino acids. Compared to Laminaria and Porphyra, essential amino acid chemical scores are higher. As a result, sargassum might make a good raw material for marine medications and dietary supplements. The nutritional profile of Sargassum sp., including its biochemical, phytochemical, and biological activity, has been thoroughly examined as a consequence. There are over 400 species of Sargassum, a genus of brown seaweed sometimes known as gulf weed or sea holly, which is a member of the Fucales order, subclass Cyclosporeae, and class Phaeophyceae. (7–9)The Sargassum genus is widely dispersed throughout the world's temperate and tropical waters. The world's tropical and subtropical regions are home to sargassum species, which are known to produce metabolites with diverse therapeutic properties, including terpenoids, polysaccharides, polyphenols, sargaquinoic acids, sargachromenol, plastoquinones, steroids, and glycerides. They typically contain terpenoids that have biological properties including the ability to damage cells, act as antioxidants, have vasodilatory effects, cause hydrozoan larvae to settle, and inhibit acetylcholine-esterase. There are various publications on their biological activity and secondary metabolites. It has been regarded as a therapeutic food of the twenty-first century due to its numerous pharmacological qualities, and research is being done on it to uncover its other pharmacological capabilities. The present study was undertaken to evaluate the effectiveness of Sargassum species in controlling human pathogen (P.mirabilis) and their antibacterial activity against Proteus mirabilis.

For usage as a herbal treatment, sargassum species are cleansed and raised. Many Chinese herbalists recommend taking 0.5 grams of powdered Sargassum—either the species S. pallidum or, less frequently, the hijiki, S. fusiforme—dissolved in warm water and consumed as a tea. The treatment for "heat phlegm" is known as hizo in traditional Chinese medicine.(10)

The Portuguese sailors who discovered sargassum in the sargasso sea gave it that name. They named it "sargaço" (Portuguese for "sargaço") after the woolly rock rose (Halimium lasianthum) that grew in their home's water wells. This genus of algae contains species that can reach lengths of several meters. They have a holdfast, stipe, and frond and are often brown or dark green in color. On particular branches, oogonia and antheridia are found in conceptacles that are integrated into receptacles. To aid in photosynthesis, certain species' fronds include berry-like gas-filled bladders that aid in flotation. Many are resistant to strong water currents because of their rough, sticky texture and sturdy, yet flexible bodies.(10,11)

MATERIALS AND METHODS:

Place of study: Department of Forensic Odontology, Saveetha Dental College, Saveetha institute of medical and technical sciences - 600 077.

Ethical clearance: Since it is in vitro study ethical clearance is not needed.

Identification of seaweed:

By examining its morphological description, existing literature references, and employing herbarium comparison studies, the Sargassum spp. identification was completed. It can be tough to discern between many Sargassum species due to the variation in form. Fortunately, the plants may be classified as belonging to the genus based on a few recurring characteristics. The genus is characterized by a strong stem-like axis and unique foliar blades. The edges of these lengthy, oval-shaped blades could be striated or smooth. There are also a lot of little berries-shaped air bladders. The colors of the plants, which range from a deep, rich chocolate to a yellow-brown, are likewise rather consistent.

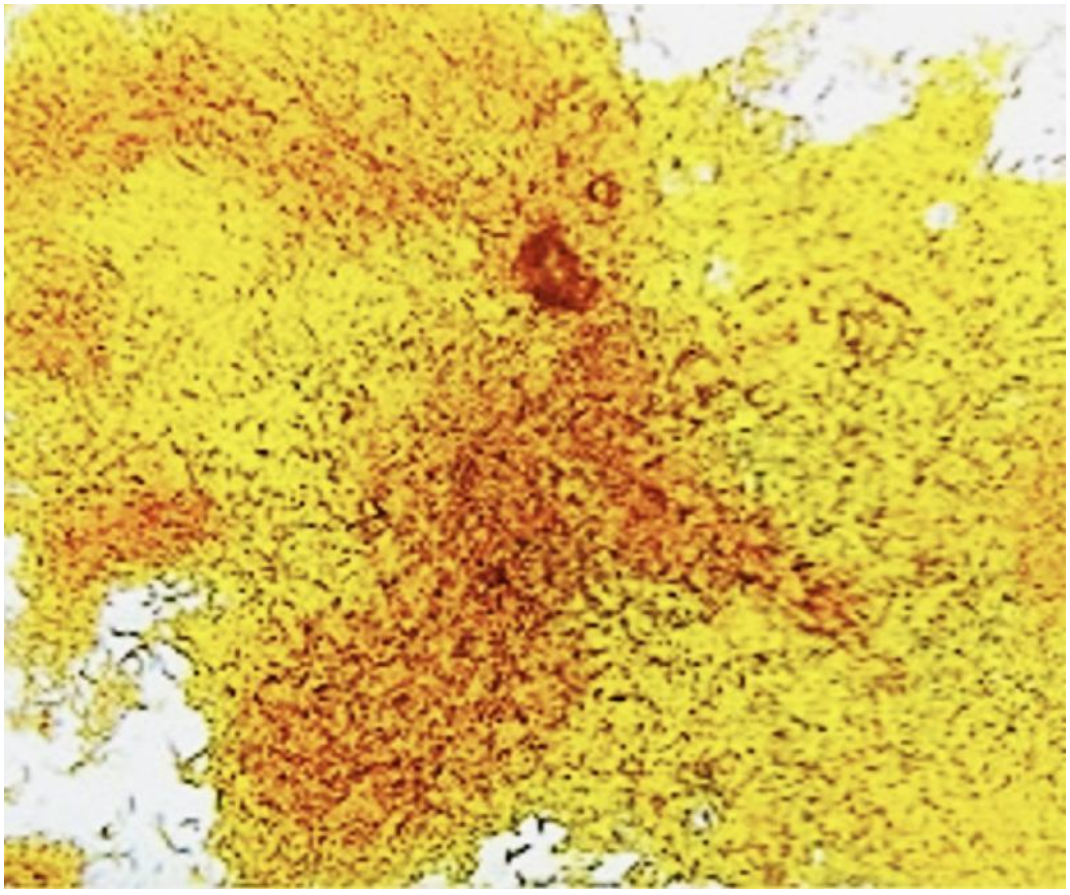
Preparation of extract:

To get rid of dirt, grit, and epiphytes, hand-picked seaweed samples were rinsed in seawater. After that, it was brought to the lab where tap water was used to clean it adequately. On blotting paper, the seaweed was then stretched out to absorb any further moisture. After being dried and powdered into a fine powder, the samples were used to create extracts.

Sargassum was dried, mixed, and then macerated with an orbital shaker for two consecutive 24-hour periods after which simplicia was administered 96% methanol (1:4). The acquired extract was next evaporated at a rate of 60 rpm at a temperature of 400 degree Celsius and it was then aerated with an aerator to produce crude extract.

Sargassum seaweed | Powdered extract of Sargassum | Methanolic extract of Sargassum

Bacterial biofilm formation:
In this investigation, 39 P. mirabilis strains were employed. They were identified from urine collected from individuals who received clinic treatment between 2011 and 2012. Twenty (51.3%) strains were identified from the urine of non-catheterized patients while 19 (48.7%) strains were isolated from the urine of catheterized patients.
Following the manufacturer's instructions, the strains were identified using VITEK GN cards (BioMérieux). The strains were kept at 70 °C in a brain-heart infusion with 20.0% glycerol (POCH) from Becton Dickinson. Two quantitative approaches were used to study the biofilm that developed in the 96-well polystyrene titer plates. Crystal violet (CV; POCH) and formazan (product of the 2,3,5-triphenyl-tetrazolium chloride dissolution, TTC; POCH) absorbance values were simultaneously studied.



Confirmation of Proteus mirabilis:
Proteus mirabilis is a gram negative stain bacteria, rod shaped bacilli with peritrichous flagellate arrangement. It is a indole negative bacterial species. It turns positive for citrate, catalase, urease and xylose test. It is negative for lactose, maltose and sucrose as well similar to indole negative aspect. The core test to distinguish Proteus from Salmonella is positive urease, and other specific tests include phenylalanine deaminase assays. One of these proteins, urease, is produced in high quantities during Proteus infections. This enzyme raises urine pH to a level where ordinarily soluble ions precipitate to produce struvite or apatite stones by catalyzing the hydrolysis of urea to carbon dioxide and ammonia. Using the enzyme urease, this test is performed to find microorganisms that can hydrolyze urea. It is frequently

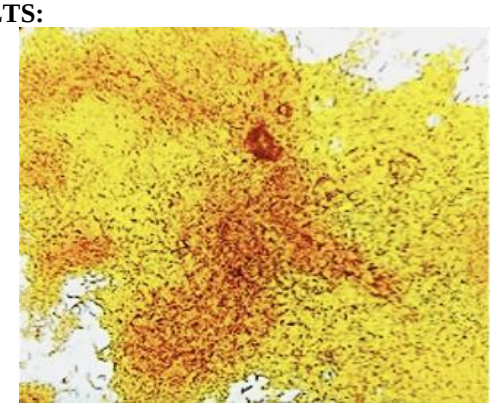
employed to set the Proteus genus apart from other intestinal bacteria. One byproduct of the hydrolysis of urea is the weak base, ammonia.

Table 1.1

Identification of Pathogen	
Gram stain	**-**
Shape	**Rod**
Motility	**+**
Indole	**-**
MR	**+**
VP	**-**
Citrate	**+**
TSI	**+**
Oxidase	**-**
Catalase	**+**
Urease	**+**
Lactose	**-**
Maltose	**-**
Sucrose	**-**
Xylose	**+**
Starch	**-**
Inosital	**-**
Genus	**Proteous**

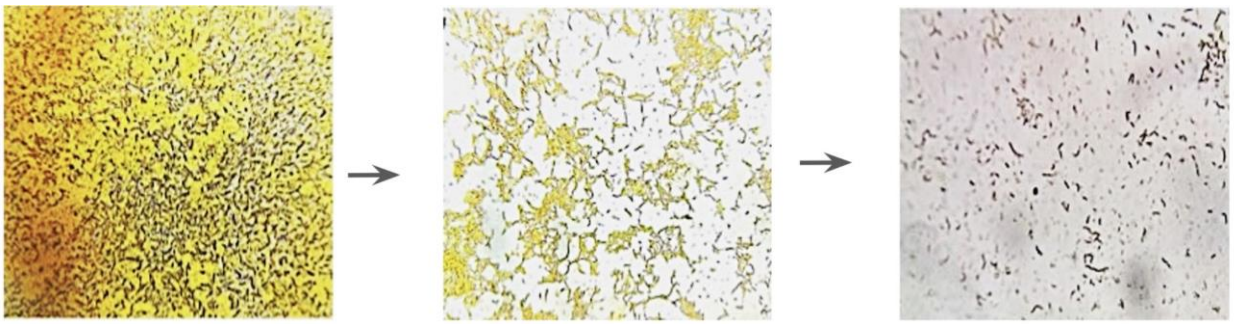
Anti bacterial activity observation:
Our sargassum extract has been utilized to treat bacteria, and the vitality of those bacteria has been assessed using confocal laser scanning microscopy (CLSM). To discriminate between live and dead bacteria on the biofilm, fluorescent dyes are used. This enables bacteria to be identified based on their cytoplasmic membrane permeability. With just one dye, confocal microscopy enables simultaneous imaging of live and dead cells and tissue coverage. Live cells are stained green by acridine orange. How much of the bead is covered with cells can be seen by comparison to the brightfield or DIC image.
Duration of the study: 3 months

RESULTS:



Untreated biofilm at initial stage
In order to distinguish between distinct cellular organelles, the fluorescent dye acridine orange intercalates or interacts with the nucleic acid (DNA or RNA) that is found in living things. The acridine molecules' electrostatic interactions with the nucleic acid and base pairs cause this binding.

Observation of live cells:
Green fluorescence when bound to dsDNA and
Red fluorescence when bound to ssDNA or RNA.
From Image 1.1 with the red fluorescence we are able to infer that our bacterial biofilm consists of live cells of P.mirabilis. Once after the treatment of our sargassum extract, we noticed decreasing levels of live cells in bacterial biofilm. We observed the declining growth of bacteria at 24, 48 and 72 hours and noticed considerable decrease in live cells of P.mirabilis in image 1.2. In our study we have only done it through in vitro methodology and found out the phenolic compound through GC-MS analysis.



Biofilm at end of 24 hours	At the end of 48 hours	At the end of 72 hours

Image 1.2 Observation of Live cells at a varying levels of 24 hours difference

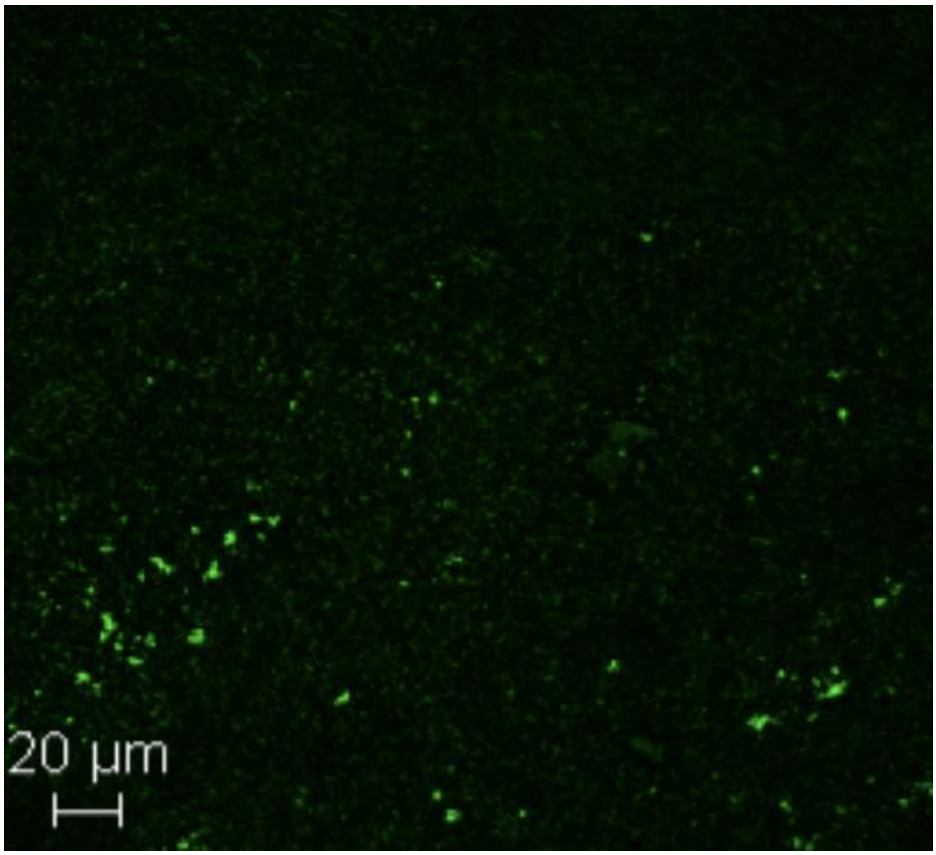
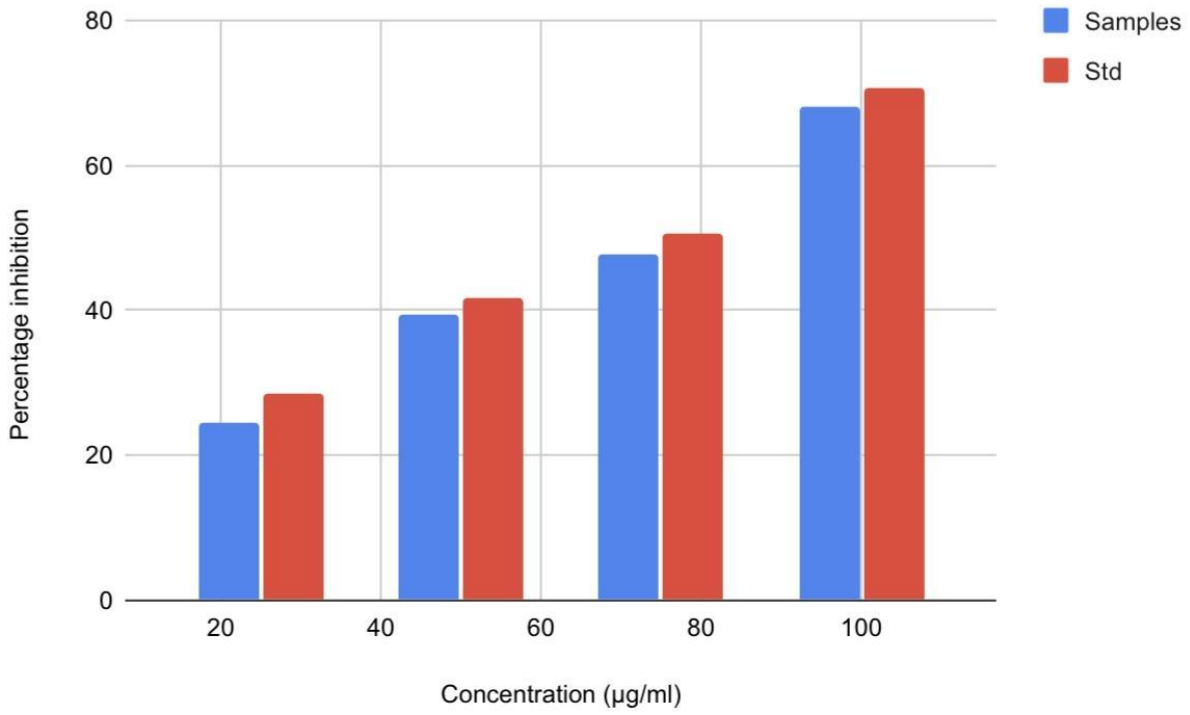


Image 1.3 Confocal images showing very few live cells

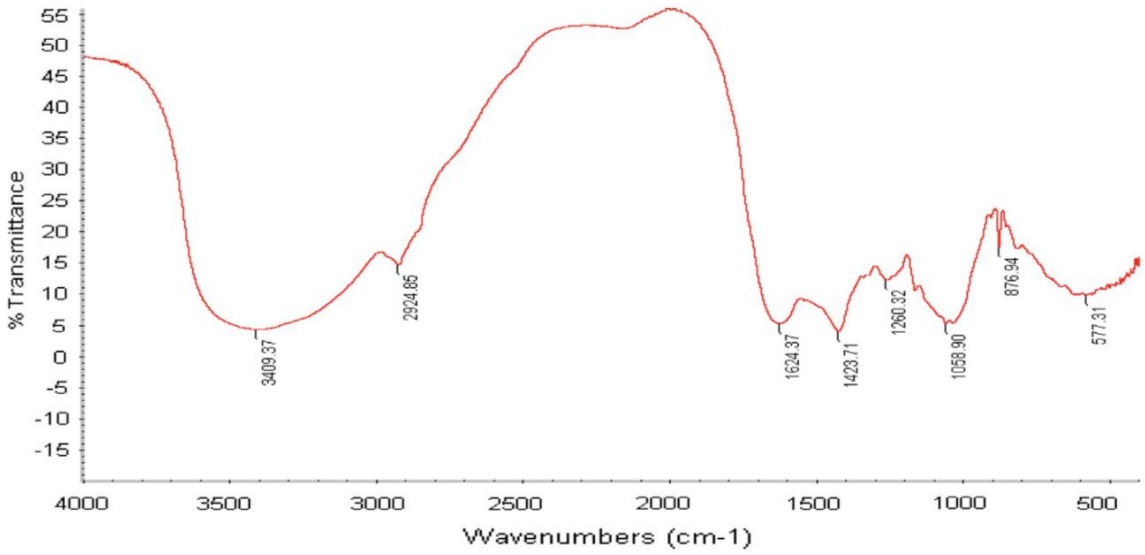
Optical slicing through tissue is possible with confocal laser scanning microscopy (CLSM). CLSM provides improved spatial resolution in living tissue and enables the viewing of living structures by removing out-of-focus pictures. The fluorescent green color enables us to confirm the exact number of Proteus mirabilis live cells present after 96 hours under the treatment with our sargassum extract.



Concentration (µg/ml)	Samples	Std
25	24.5	28.5
50	39.4	41.8
75	47.8	50.7
100	68.2	70.7

Table 1.2

The standard antibiotic which we used for our study is ampicillin which is currently used antibiotic against P.mirabilis species. The antibacterial capacity of seaweed extract that is methanolic extract of sargassum was assessed using the by gauging how well they were able to inhibit growth of P.mirabilis species, Evaluation of antibacterial activity at 25 and 50 microgram/mL of standard drug revealed 28.5% and 41.8%, respectively (Table 1.2). When the concentration of methanolic extract of sargassum was increased, inhibition of P.mirabilis biofilm were increased at concentrations of 25 microgram/mL (p 0.05). Between the extract concentrations of 25 microgram/mL (24.5%) and 30 microgram/mL (26.2%) (there was no difference (ns#). The methanolic extract of sargassum has a maximum antibacterial activity of 68.2% at a concentration of 100 microgram/mL. Standard drug at the same concentration (100microgram /mL) shows 70.7% antibacterial property against P.mirabilis species.
The FTIR analysis of the methanolic antibacterial active ingredient from U. lactuca revealed the existence of 4 peaks with various peak relative intensities and retention times, as shown in image 1.3



The strongest peak in the FTIR analysis of Sargassum indicates that phenol is the main component in this seaweed extract.

DISCUSSION:

The pharmaceutical industry is interested in the potential bioactive molecules that marine creatures may produce since they are a rich source of novel and physiologically active metabolites. Different algae have been demonstrated to have in vitro antibacterial action against gram positive and gram negative bacteria in extracts and components. According to their composition and concentration, phenol compounds—supposed to be the primary chemical constituents of algal cells—could either activate or inhibit microbial growth (Alberto et al., 2001; Reguant et al., 2000). Since some bioactive metabolites found in the algal materials have the ability to be soluble in one solvent but not in another, the efficacy of this activity may also rely on the solvents being employed as well as the type of seaweeds. According to Cox et al. (2010), the extraction of antimicrobials from various species depended on the kind of solvent used; metha- nol was a good solvent for extracting antimicrobials from brown seaweeds, whereas acetone was better for extracting antimicrobials from green species. Methanolic seaweed extracts produced greater antimicrobial activity, according to Manilal et al. (2009) and Rangaiah et al. (2010); Viachosi et al. (2001) reported that extracts of the Phaeophyta displayed the highest level of antibacterial activity, followed by the Rhodophyta and then the Chlorophyta. All of these earlier findings confirmed our findings; stronger antibacterial activity was obtained with the methanol brown seaweed Sargassum species, according to Kandhasamy and Arunachalam's (2008) observation that brown and green algae were more active than other kinds of marine macroalgae. (12)

Our findings also showed that a strong inhibitory effect was precisely detected on P.mirabilis species. The outer membrane of gram negative bacteria serves as a barrier to a variety of environmental elements, including antibiotics (Tortora et al.,

2001). This high inhibition suggests that a potent medicine from marine natural sources could be developed and used to treat human diseases. In addition, the way the seaweed extracts were made, when they were made, and where they were collected all have a significant impact on how effective they are (Padmini Sreenivasa Rao et al., 1986). In comparison to fresh extracts, dried seaweeds showed higher antimicrobial activity; lyophilization of algal samples results in a greater compound extraction; in contrast, fresh seaweeds, which have a higher water content, produce diluted extracts with lower or even negligible inhibitory activity. According to FTIR Chemical analysis, the main constituents, especially in Sargassum species, were of a phenolic type, which is linked to powerful antibacterial properties (Reguant et al., 2000; Alberto et al., 2001). The greatest absorption of hydroxyl amide groups, relative to phenol molecules, ranges between 3421 cm1, which indicates this.

Bacterial Species	Proteus mirabilis		
Virulence gene	Adhesion	Urease	Hemolysin
Primer	UcaAF UcaAR	UreCF UreCR	HpmAF HpmAR
Amplified PCR products	1	2	1
size of PCR products	780bp	650bp	375bp

The virulence genes (adhesion, hemolysin, and urease) of the tested multidrug resistant UTI bacterial isolates (P. mirabilis) were found using three distinct primer pairs (UcaAF UcaAR; UreCF UreCR; and HpmAF HpmAR). The pathogenicity of UTIs can be ascribed to a number of virulence factors (Tarchouna et al., 2013). The virulence genes causing multidrug resistance in the UTI bacterial isolates were examined using the Polymerase Chain Reaction method. (13)Three virulence genes in the bacterial isolate, including ucaA (adhesion), ureC (urease), and hpmA (hemolysin), were specifically targeted with specific primers. The colonization of bacteria in the urinary system is caused by these three genes. Due to the synthesis of the urease enzyme, the ureC gene is thought to be a diagnostic marker for the presence of UTI bacteria. The findings revealed that 96.66% of this P.mirabilis had this gene. (13–15)

It was discovered that the presence of the virulence genes (adhesion, urease, and hemolysin) is an essential component for the successful colonization of the uropathogenic bacteria and its multidrug resistance property. This information was obtained by relating the results of the antibacterial activities of sargassum extract with those of the PCR of the studied UTI bacteria P.mirabilis species. By reducing the activity of the virulence genes and preventing (as in the case of P. mirabilis) infection, chemicals produced from seaweed have demonstrated their potential as new natural sources for antibiotics.(16)

One of the largest producers of biomass, marine algae have also shown promise as a source of novel and distinctive chemicals for a variety of uses. (17)A large number of seaweed-derived substances exhibit bacteriostatic or antibacterial, antiviral, anticancer, anti-inflammatory, and antifouling properties. Sargassum was processed into an aromatic ester derivative in this study utilizing methanol. A phenolic bioactive substance with promising antibacterial properties against multidrug-resistant UTI pathogenic bacteria, was chemically discovered. In further studies the exact name of the phenolic compound should be confirmed and its mechanism of action should be studied. As a result, the study suggests using seaweeds as potential remedies for human illnesses or as new antibacterial agents to take the place of synthetic antibacterial medications.(18)

CONCLUSION:

The presence of phenols, the main chemical constituent correlated to high antibacterial activity, along with a variety of algal species, variable solvents, and different extraction techniques all combine to make seaweeds a potent antimicrobial agent, replacing synthetic chemical products and opening up new, promising horizons for the pharmaceutical industry. In the present investigation methanolic extract of sargassum showed a promising antibacterial effect against the biofilm of Proteus mirabilis.

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4. Kumaran Department Stores

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CONFLICT OF INTEREST

The authors hereby declare that there is no conflict of interest in this study.

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