



Antibacterial Activity of Silver Nanoparticles of Seaweeds

B.Vasudeva Rao* and Dr. M. Boominathan

Department of Biotechnology, Marudupandiyar College, Thanjavur -613 403, Tamilnadu, India

Date of Receipt- 17/11/2015
Date of Revision- 06/12/2015
Date of Acceptance- 25/12/2015

Address for Correspondence

Department of
Biotechnology,
Marudupandiyar
College, Thanjavur -
613 403, Tamilnadu,
India

Tel. : +91-9010555556

E-mail: vasu.balagam@gmail.com

ABSTRACT

In the present study, antibacterial activity of silver nanoparticles synthesized from the seaweeds of *Ulothrixflacca*, *Ulva fasciata* and *Caulerpataxifolia* were analyzed against four gram positive and gram negative bacterial strains of common fish and human pathogens. As these pathogens cause enormous damage to aquaculture as well as pose harmful threat to humans and their emergence as multidrug-resistant strains is a major problem in the control of infections.

Further antibacterial activity of silver nanoparticles prepared from seaweeds against eight pathogenic strains was determined by agar well diffusion assay and MIC was estimated by qualitative experimentation by micro broth dilution method. IC₅₀ values were also studied using spectrophotometry. All experiments were done in triplicate.

The silver nanoparticles of *Ulothrixflacca*, *Ulva fasciata* and *Caulerpataxifolia* showed the zone of inhibition ranges from 0.7± 0.6 to 26.3±0.6mm. The average IC₅₀ values of the methanol extracts of *Ulva fasciata*, *Caulerpataxifolia* and *Ulothrixflacca* are 57µg/ml, 61µg/ml and 59µg/ml respectively.

Silver nanoparticles from *Ulothrixflacca*, *Ulva fasciata* and *Caulerpataxifolia* possess very good antibacterial activity which makes them a potent source of antibacterial agent.

Keywords: Antibacterial activity, Silver nanoparticles, Seaweeds, MIC, IC₅₀, Fish and Human pathogens.

INTRODUCTION

Marine algae produce pharmacologically important diverse group of natural products^{1,2} that include algae, which produce novel and unexplored sources of potentially useful bioactive compounds that

might represent useful leads in the development of new Pharmaceutical agents³.

Bacteria are common disease agents, as shown by the wide clinical use of antibiotics. However, a new global health

problem has arisen as indiscriminant antibiotic use and the remarkable ability of bacteria to acquire resistance (via genetic mutation or gene acquisition) lower these drugs' effectiveness. Clearly, new classes of antibiotics with novel structures are needed to combat this trend.

The antimicrobial activity of microalgae extracts is generally assayed using various organicsolvents⁴. An organic solvent always provides a higher efficiency in extracting compounds for antimicrobial activity as compared to aqueous extract^{5,6}. Marine algae are a rich source of novel bioactive compounds which may find several applications^{7,8}. Secondary or primary metabolites from these organisms may be potential bioactive compounds of interest for the pharmacological industry. The cell extracts and active constituents of various algae have been shown to have antibacterial activity against Gram positive and Gram negative bacteria. The production of bioactive metabolites is considered to be a response to ecological pressures such as competition, predation deterrence, and reproduction. Such chemicals are often common in sessile eukaryotic organisms like marine sponges and corals, seaweeds, and terrestrial plants^{9,10}. Silver nanoparticles (Ag-NPs) have been known for its inhibitory and bactericidal effects in the past decades¹¹. Antibacterial activity of silver containing materials can be applied in medicine for reduction of infections on the burn treatment^{12,13}. AgNPs have been synthesized using marine microalgae and screened for its antibacterial activity¹⁴. Noble metal nanoparticles show unique electronic, optical, magnetic and chemical properties, which differ considerably from those of the corresponding bulk materials¹⁵⁻¹⁷ and hence preparations of noble metal nanoparticles are of technological importance. Recently inorganic nanoparticles protected by organic

ligands have attracted much interest due to their diverse technological applications¹⁸⁻²⁰.

Seaweeds contain various organic and inorganic substances which can benefit human health²¹. Due to the high amount of vitamins and minerals present in the green seaweed it is used widely in agriculture, pharmaceutical, biomedical, and nutraceutical industries²². About 250 macro algal species have been commercially utilized worldwide and about 150 species are favorably consumed as human food²³.

Seaweeds concentrate minerals and trace elements from marine water and convert them in organic forms as they grow in a mineral-rich medium²⁴. The numerous elements coming from the sea are Ca, Cl, Cu, I, Mg, Mn, Na, P, S and Zn²⁵. They selectively absorb elements like Na, K, Ca, Mg, I and Br from the seawater and accumulate them in their thalli. The accumulated elements vary from species to species. For example, large quantities of K and I are taken up by many brown seaweeds and Ca and Br by red algae. The possibility of free-radical involvement in the antibacterial activity of silver nanoparticles (Ag-NPs) has been previously reported²⁶, but the underlying mechanism and characteristics remain unclear. Silver ions cause the release of K⁺ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes and DNA, is an important target site of silver ions²⁷⁻³⁰. After all intensive studies on the antibacterial activities on various kinds of seaweeds, chlorophyceae members showed well antibacterial activity on both gram positive and gram negative bacteria.

In the present study the antibacterial activity of seaweeds (*Ulothrixflacca*, *Ulva fasciata* and *Caulerpataxifolia*) extracts was studied against eight common pathogens of fish and human.

MATERIALS AND METHODS

Seaweed collection

Ulothrixflacca, *Ulva fasciata* and *Caulerpataxifoliasee*weeds collected from the rocky platforms at tenneti park located at Vishakhapatnam coastal banks. Species identification was done by referring the printed journal (Y. Sarojini, P.SanthaRao, B. Sujatha K.Lakshminarayana Distribution and diversity of marine macro algae in relation to environmental factors at Visakhapatnam coast. Seaweed Res. Utiln., 35 (1&2): 55-64, 2013 and authenticated at Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh.

The three seaweed species were collected during the months of July and August of the year 2013. They were carefully picked from the submerged rocks of Vishakhapatnam coastal banks and washed with fresh water thoroughly to remove the cell debris, sand and other particles. They were then kept in the ice bags and transferred to the laboratory. In laboratory, seaweeds were again washed with distilled water carefully and kept for shade dry. After drying, the seaweeds were stored in desiccator for further use.

Preparation of silver nanoparticles

1gm of each seaweed powder was extracted with methanol using soxhlet and filtered. 1mM Silver nitrate solution was added to the filtrates slowly under magnetic stirring conditions for even coating of silver and subjected to heating at 12°C for 10 min. The extracts are used as reducing and stabilizing agent for 1mM of Silver nitrate. This one pot green synthesis was the modified method followed by Vigneshwaran et al.³¹.

Pathogen selection

The increasing resistivity of several pathogens against the present antibiotics insists the need for the new molecules to

fight against those pathogens. Especially in aquaculture, the pathogenic bacteria cause immense damage to the pond. Of all the aqua pathogenic bacteria, very few shows pathogenicity over humans. Such kind of pathogens which uses both aqua species and humans as their hosts were selected for the present study. Four gram positive and four gram negative pathogens were procured from MTCC Chandigarh.

Evaluation of antimicrobial activity of the bio-synthesized silver nanoparticles

The prepared algal extracts (*Ulothrixflacca*, *Ulva fasciata* and *Caulerpataxifolia*) were tested for Minimum Inhibitory Concentration (MIC), IC50 values and antibacterial activity by well diffusion method against pathogenic bacterial strains.

Test organisms

Sl. no	Organism	Code	Type
1	<i>Staphylococcus aureus</i>	MTCC 3160	gm positive
2	<i>Listeria monocytogenes</i>	MTCC 839	gm positive
3	<i>Clostridium perfringens</i>	MTCC – 450	gm positive
4	<i>Mycobacterium spp</i>	MTCC – 290	gm positive
5	<i>Delftiaacidovorans</i>	MTCC- 104	gm negative
6	<i>Edwardsiellatarda</i>	MTCC-2400	gm negative
7	<i>Plesiomonasshigelloides</i>	MTCC-1737	gm negative
8	<i>Vibrio vulnificus</i>	MTCC- 1145	gm negative

The pathogenic cultures were subcultured into peptone broth and incubated at 37°C to attain 10⁵-10⁶ CFU/ml using MacFarland's standard and were used in further experiments.

Agar well-diffusion method

Muller Hinton Agar plates were swabbed with fresh cultures of pathogenic organisms using sterile cotton swab. Approximately 6-mm diameter of well was made with the help of sterile gel puncture.

Using a micropipette, the prepared algal extracts were loaded at different concentrations. Ampicillin antibiotic (50µg/ml) was used as a positive control, followed by incubation at 37°C for 18-24 h. After incubation effect of nanoparticles on tested bacterial culture were determined by measuring the zone of inhibition compared with control.

RESULTS

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation. IC50 value is the concentration of the extract required for 50% growth inhibition. The pathogenic cultures were subcultured into peptone broth and incubated at 37°C to attain 10^5 - 10^6 CFU/ml using MacFarland's standard and were used in further experiments.

According to the IC50 values that are shown in the (Table-1), the average IC50 values of the methanol extracts of *Ulva fasciata*, *Caulerpataxifolia* and *Ulothrixflacca* are 15µg/ml, 27µg/ml and 23µg/ml respectively. So these three respective concentrations were taken to study the zone of inhibition assay for all the eight pathogens (Table -2). Of all the three chlorophyta members, *caulerpataxifolia* showed maximum zone of inhibition activity when compared with the other two. On the whole the antibacterial activity is shown significantly more on the gram negative bacteria than the gram positive bacteria. Gram negative bacteria *Vibriovulnificus* and *Plesiomonasshigelloides* showed resistance against *ulothrixflacca* with no zone of inhibition (shown in graph-9).

DISCUSSION

The inhibitory action of silver compounds and silver ions had been

historically recognized and applied as a useful therapeutic agent for preventing wound infections. The inhibitory action of silver on bacterial cells is related to the strong interaction of silver with thiol groups present in key respiratory enzymes in bacteria³². Whereas, a crystalline silver shows the most effective inhibitory action with a rapid inhibition rate³³. The biosynthesized nanoparticles also exhibit well significant antibacterial activity. *Ulva fasciata* silver nanoparticles (57µg/ml) showed highest zone of inhibition (26.3±0.6mm) against *Listeria monocytogenes*. It was observed that *Ulothrixflacca* showed no activity on *Plesiomonasshigelloides*. *Caulerpataxifolia* showed significant activity on all the eight pathogens. The present study showed the gram positive microorganism was more susceptible to the extracts of the algae used. Tuney et al. (2006) also reported that Gram-positive bacteria were more effectively controlled by the extracts of algae used in their study compared to Gram-negative bacteria³⁴. Similar observations were made by Taskin et al. (2001)³⁵ and (Paz et al., 1995)³⁶ indicating that the more susceptibility of Gram-positive bacteria to the algal extract was due to the differences in their cell wall structure and their composition. The presence of thick murine layer in the cell wall also prevents the entry of the inhibitors (Martin, 1995)³⁷. In Gram-negative bacteria, the outer membrane acts as a barrier to many environmental substances including antibiotics (Tortora et al., 2001)³⁸. The present study showed the use of biosynthesized nanoparticles to control several pathogens that affect the aquaculture economy. The active compounds in this activity need to be isolated and characterized to identify potential pharmaceutical applications for the bioactivity results reported in the present study. More studies regarding the application of these active compounds to the aquaculture gives better

results in disease control and management. As the seaweeds are rich in various micro elements and minerals they can be administered to the aquatic environment. Among the three sea weeds *Caulerpataxifolia* exhibited better antibacterial activity when compared to the other two seaweed extracts.

CONCLUSION

From the present study we conclude that even at very small concentration (in µg/ml) AgNPs of three seaweeds (*Ulva fasciata*, *Caulerpataxifolia* and *Ulothrixflacca*) possess very good antibacterial activity which makes them a potent source of antibacterial agents against the common pathogens of fish and human. Of all three seaweeds *Caulerpataxifolia* exhibited significant activity when compared with the other two compounds. Also, synthesis of AgNPs can potentially eliminate the problem of chemical agents that may have adverse effects, thus making nanoparticles more compatible with the eco-friendly approach. Moreover the synthesized AgNPs enhance the therapeutic efficacy and strengthen the medicinal values of *Ulva fasciata*, *Caulerpataxifolia* and *Ulothrixflacca*. Hence, our results are promising and prove to be an important step in this direction as it decreases the load of multidrug resistant pathogens in aquaculture.

ACKNOWLEDGEMENT

We thanks to management of Department of Biotechnology, Marudupandiyar College, Thanjavur -613 403, Tamilnadu, India for providing experimental facilities.

REFERENCES

1. Ravikumar S, Krishnakumar S, Jacob Inbaneson S, Gnanadesigan M Antagonistic activity of marine

actinomycetes from Arabian Sea coast. *Archives of Applied Science Research*. 2010; 2(6):273-280.

2. Krishnakumar S, Premkumar J, Alexis Rajan R, Ravikumar S Optimization of potential antibiotic production by salt-tolerant actinomycetes *Streptomyces sp.* - MSU29 isolated from marine sponge. *International J on Applied Bioengineering*. 2011; 5(2):12-17.
3. Iwamoto C, Yamada T, Ito Y, Minoura K, Numata A Cytotoxic cytochalasins from a *Penicillium* species separated from a marine alga. *Tetrahedron*. 2001; 57: 2904–2997.
4. Cordeiro RA, Gomes VM, Carvalho AFU, Melo VMM Effect of Proteins from the Red Seaweed *Hypneamusciiformis* (Wulfen) Lamouroux on the Growth of Human Pathogen Yeasts. *Brazilian Arch Boil Technol*. 2006; 49(6): 915-921.
5. Masuda M, Abe T, Sato S, Suzuki T, Suzuki M Diversity of halogenated secondary metabolites in the red alga *Laurencianipponica* (Rhodomelaceae, Ceramiales). *J of Phycology*. 1997; 33: 196-208.
6. Lima-Filho JVM, Carvalho AFFU, Freitas SM, Melo VMM Antibacterial activity of extracts of six macroalgae from the northeastern Brazilian coast. *Brazilian J of Microbiology*. 2002; 33: 311-313.
7. Aziz A, Poinssot B, Daire X, Adrian M, Bezier A, Lambert B, Joubert JM, Pugin A (2003). Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Mol. Plant microbe interact*. 16:1118-1128.
8. Delattre C, Michaud P, Courtois B, Courtois J, (2005). Oligosaccharides engineering from plants and algae applications in biotechnology and

- therapeutics. *Minerva Biotec.* 17:107-117.
9. Rosenthal GA & DH Janzen. 1979. *Herbivores: Their interaction with secondary plant metabolites*, 718 pp. Academic Press, Orlando.
 10. Pawlik JR. 1993. Marine invertebrate chemical defenses. *Chemical Reviews* 93: 1911-1922.
 11. Cho KH, Park JE, Osaka T, Park SG: The study of antimicrobial activity and preservative effects of nanosilver ingredient. *ElectrochimActa* 2005, 51:956–960.
 12. Ulkur E, Oncul O, Karagoz H, Yeniz E, Celikoz B: Comparison of silvercoated dressing (Acticoat™), chlorhexidine acetate 0.5% (BactigrassW), and fusidic acid 2% (FucidinW) for topical antibacterial effect in methicillin-resistant staphylococci-contaminated, full-skin thickness rat burn wounds. *Burns* 2005, 31:874–877.
 13. Parikh DV, Fink T, Rajasekharan K, Sachinvala ND, Sawhney APS, Calamari TA, Parikh AD: Antimicrobial silver/sodium carboxymethyl cotton dressings for burn wounds. *Text Res J* 2005, 75:134–138.
 14. Merin DS, Prakash, Bhimba VB (2010) Antibacterial screening of silver nanoparticles synthesized by marine microalgae. *Asian Pac J Trop Med* 3: 797- 798. 10. Hameed SV, Sult.
 15. D. L. Feldheim and C. A. Foss, “Metal nanoparticles: Synthesis, Characterization and Applications,” Marcel Dekker Inc., New York, 2002.
 16. G. Cao, “Nanostructures and Nanomaterials,” Edited by Imperial College Press, London, 2004.
 17. C. P. Poole and F. J. Owens, “Introduction to Nanotechnology,” Edited by Wiley Interscience Publication, New Jersey, 2005.
 18. M. Brust, M. Walker, D. Bethell, D. J. Schiffrin and R. J. Whyman, “Synthesis of Thiol Derivatized Gold Nanoparticles in a Two Phase Liquid/Liquid System,” *Journal of the Chemical Society, Chemical Communications*, Vol. 7, No. 7, 1994, pp. 801-802. doi:10.1039/c39940000801.
 19. A. S. Nair and T. Y. Pradeep, “Halocarbon Mineralization and Catalytic Destruction by Metal Nanoparticles,” *Current Science*, Vol. 84, No. 12, 2003, pp. 1560-1564.
 20. Y. Fang, “Optical Absorption of Nanoscale Colloidal Silver: Aggregate Band and Adsorbate-Silver Surface Band,” *Journal of Physical Chemistry*, Vol. 108, No. 10, 1998, pp. 4315-4318. doi:10.1063/1.475831 [7] X. Wang, J. Zhuang, Q. Peng and Y.
 21. S. Cox, N. Abu-Ghannam and S. Gupta, An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds, *Int. Food Res. Journal.* 17 (2010) 205-220.
 22. M. Padua, P. Segio, Growoski Fontoura and A. Luiz Mathias, Chemical Composition of *Ulvariaoxysperma*(Kützing) Bliding, *Ulva lactuca*(Linnaeus) and *Ulva fascita*(Delile), *Braz. Arch. of Biol and Technol.* 47 (1) (2004) 49-55.
 23. P. Kumari, M. Kumar, V. Gupta, C.R.K. Reddy and B. Jha, Tropical marine macro algae as potential sources of nutritionally important PUFAs. *Food Chem*, 120 (2010) 749–757.
 24. Chapman, V.J. and D.J. Chapman, 1980. *Seaweeds and Their Uses*, 3rd edn. Chapman and Hall, New York, pp: 62-96.
 25. Jarvis, D.C., 1976. *Folk Medicine: A Doctor's guide to good health*. London, Pan Books, pp: 120-132.
 26. Kim, J. S., E. Kuk, K. N. Yu, J.-H. Kim, S. J. Park, H. J. Lee, S. H. Kim, Y. K.

- Park, Y. H. Park, *et al.* 2007. Antimicrobial effects of silver nanoparticles. *Nanomed-Nanotechnol.* 3: 95- 101.
27. Fuhrmann, G. F. and A. Rothstein. 1968. The mechanism of the partial inhibition of fermentation in yeast by nickel ions. *Biochim. Biophys. Acta.* 163: 331-338.
28. Miller, L. P. and S. E. A. McCallan. 1957. Toxic action of metal ions to fungus spores. *J. Agric. Food Chem.* 5: 116- 122.
29. Rayman, M. K., T. C. Lo, and B. D. Sanwal. 1972. Transport of succinate in *Escherichia coli*. II. Characteristics of uptake and energy coupling with transport in membrane preparations. *J. Biol. Chem.* 247: 6332-6339.
30. Schreurs, W. J. and H. Rosenberg. 1982. Effect of silver ions on transport and retention of phosphate by *Escherichia coli*. *J. Bacteriol.* 152: 7-13.
31. Vigneshwaran N, Nachane RP, Balasubramanya RH, Varadarajan PV (2006) A novel one-pot 'green' synthesis of stable silver nanoparticles using soluble starch. *Carbohydr Res* 134: 2012-2018. 19. Chandran SP, Chaudhary M, Pasricha R, Ahmad
32. Gordon O, VigSlenters T, Brunetto PS, Villaruz AE, Sturdevant DE, *et al.* (2010) Silver coordination polymers for prevention of implant infection: thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. *Antimicrob Agents Chemother* 54: 4208-4218.
33. Wright JB, Lam K, Burrell RE (1998) Wound management in an era of increasing bacterial antibiotic resistance: a role for topical silver treatment. *Am J Infect Control* 26: 572-577.
34. Tuney I, Cadirci BH, Unal D, Sukatar A (2006). Antimicrobial activities of the extracts of marine algae from the Coast of Urla (zmir, Turkey). *Tur. J. Biol.* 30: 1-5.
35. Taskin E, Ozturk M, Kurt O (2001). Antibacterial activities of some marine algae from the Aegean Sea (Turkey). *Afr. J. Biotechnol.* 6: 2746-2751.
36. Paz EA, Lacy RN, Bakhtian M (1995). The B-Lactum antibiotics penicillin and cephalosporin in perspective. Hodder strong, London.p. 324.
37. Martin GJ (1995). *Ethnobotany: A Methods Manual*, Champan and Hall, London.
38. Tortora GJ, Funke BR Case CL (2001). *Microbiology: An Introduction*. Benjamin Cummings. San Francisco, p. 88.

Table 1. Minimum Inhibitory Concentration and IC50 values of AgNP's of seaweeds against pathogens

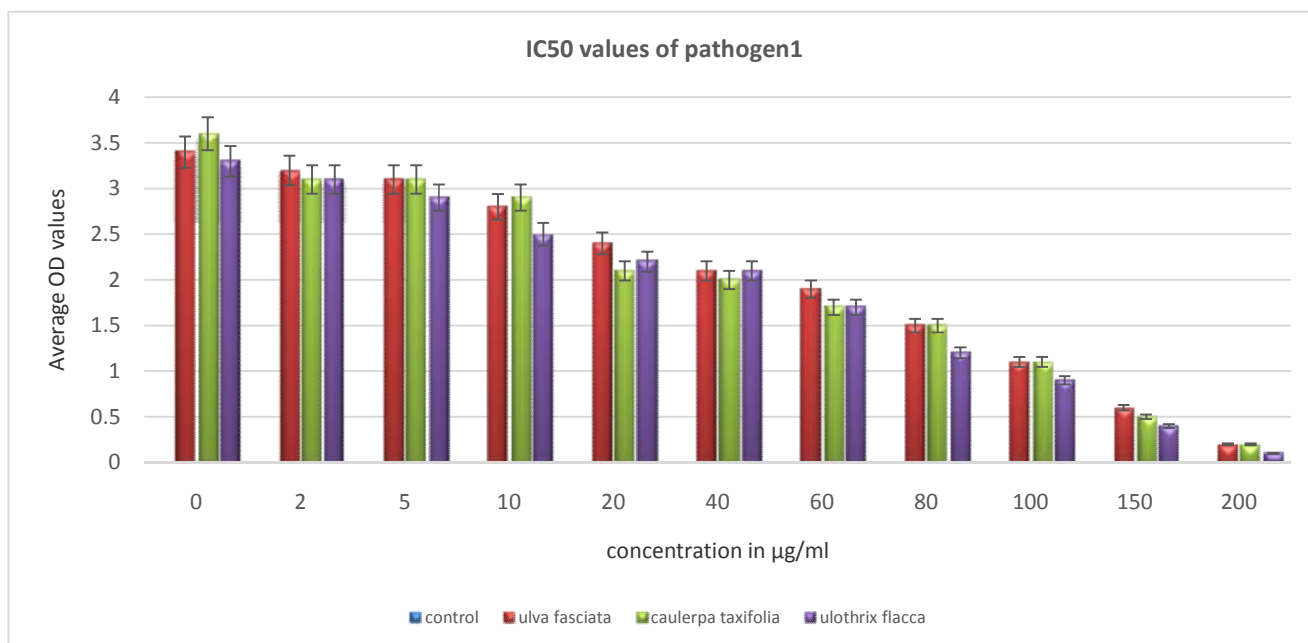
Species selected	Caulerpataxifolia (µg/ml)		Ulva fasciata (µg/ml)		Ulothrixflacca (µg/ml)	
	MIC	IC50	MIC	IC50	MIC	IC50
<i>Mycobacterium spp</i>	200	70	200	80	180	60
<i>Clostridium perfringens</i>	200	80	200	80	200	80
<i>Listeria monocytogenes</i>	200	80	150	60	200	80
<i>Staphylococcus aureus</i>	150	40	100	40	150	40
<i>Vibrio vulnificus</i>	200	60	200	60	200	60
<i>Plesiomonasshigelloides</i>	150	60	100	40	150	60
<i>Edwardsiellatarda</i>	150	50	100	40	100	50
<i>Delftiaacidovorans</i>	150	50	150	60	100	40

Measurement of zone of inhibition

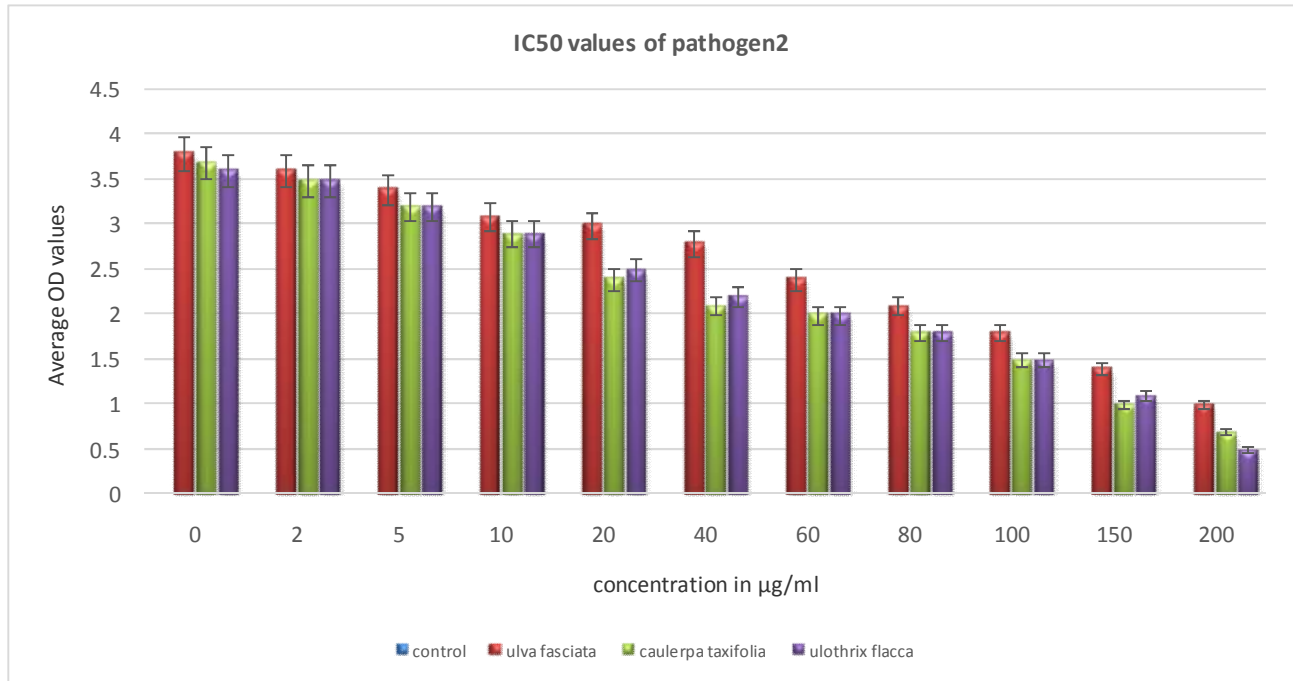
Table 2. Antibacterial activity [Zone of Inhibition in mm (mean±SD)] of AgNP's of *Caulerpataxifolia*, *Ulvafasciata*, and *Ulothrixflacca* against pathogens

Species tested	AgNP's of methanol extracts Mean±SD values			
	Caulerpataxifolia	Ulva fasciata	Ulothrixflacca	Positive control Ampicillin
<i>Mycobacterium spp</i>	14±1.0	17.3±0.6	13.6±0.6	12.3±0.2
<i>Clostridium perfringens</i>	19.6±0.6	19.3±0.6	18±1.0	11.4±0.6
<i>Listeria monocytogenes</i>	20.6±0.6	26.3±0.6	17±1.0	13.5±0.3
<i>Staphylococcus aureus</i>	16.6±0.6	15.6±1.5	13.6±0.6	10.5±0.5
<i>Vibriovulnificus</i>	10±1.0	8.6±0.6	0.7±0.6	12.15±0.6
<i>Plesiomonasshigelloides</i>	7.7±0.6	0.7±0.6	0±0	09.4±0.2
<i>Edwardsiellatarda</i>	14.3±1.5	15.6±0.6	16±1.0	10.4±0.2
<i>Delftiaacidovorans</i>	14.7±0.6	14.3±1.1	10±1.0	11.5±0.6

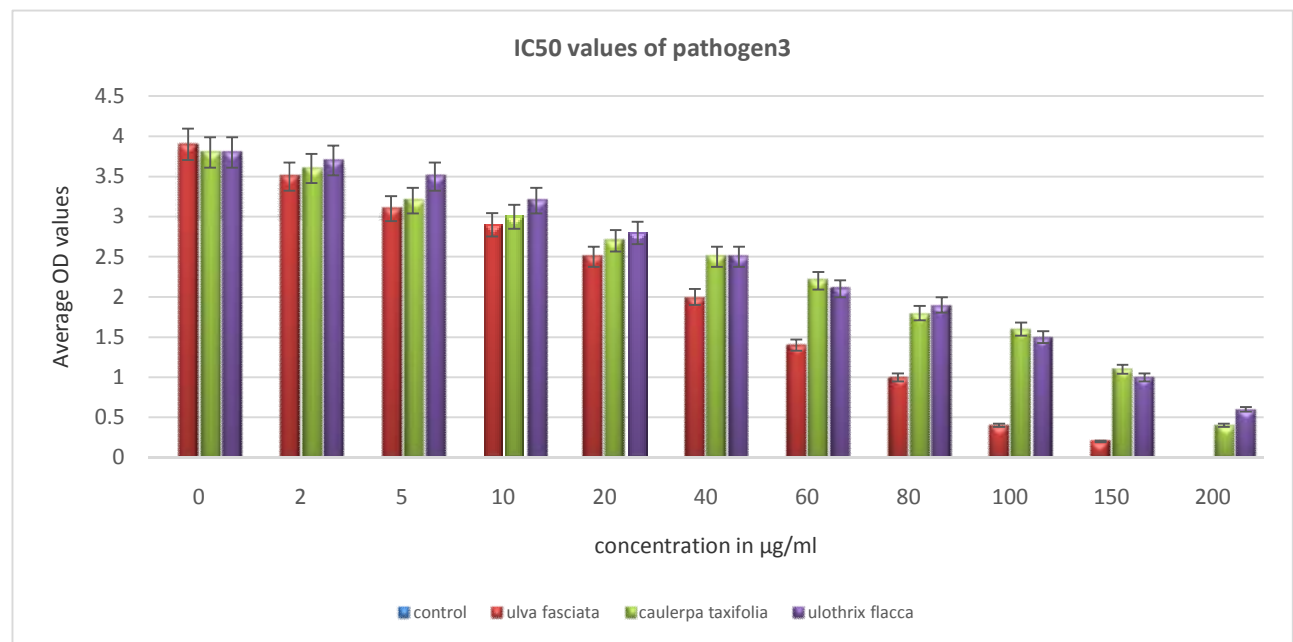
The IC₅₀ values of the eight pathogens are calculated as per the Optical Density values and shown in the graphs below.



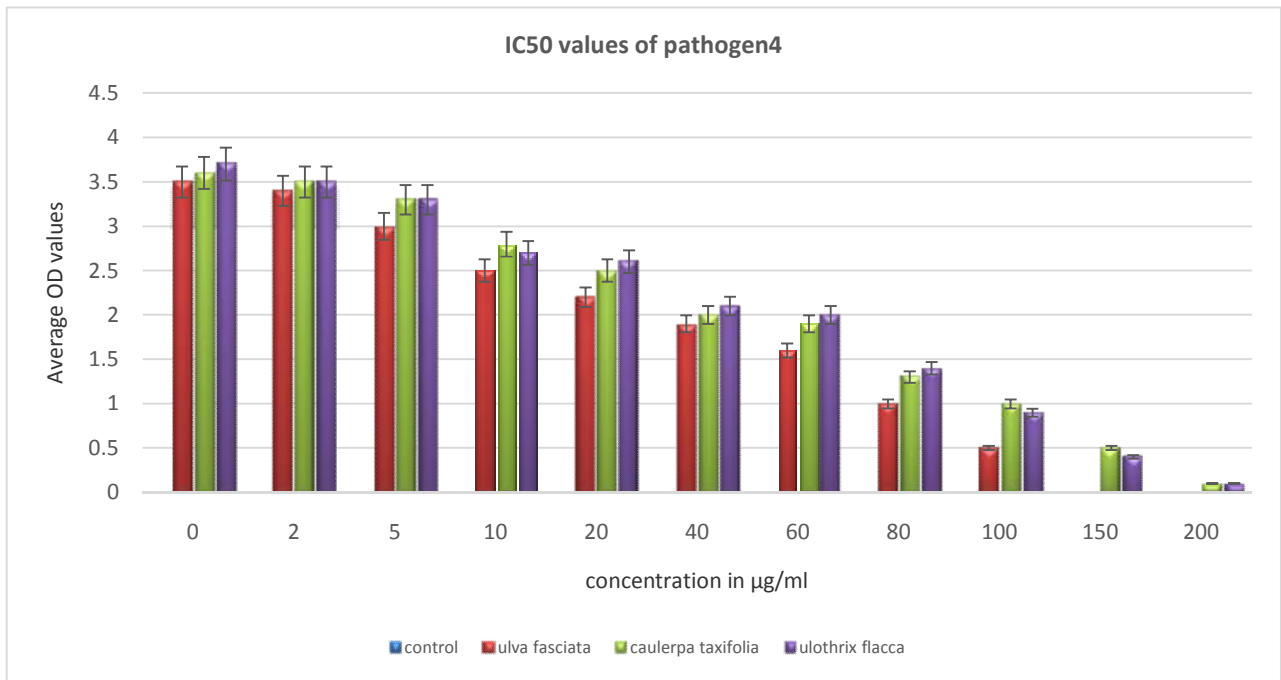
Graph 1. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-1



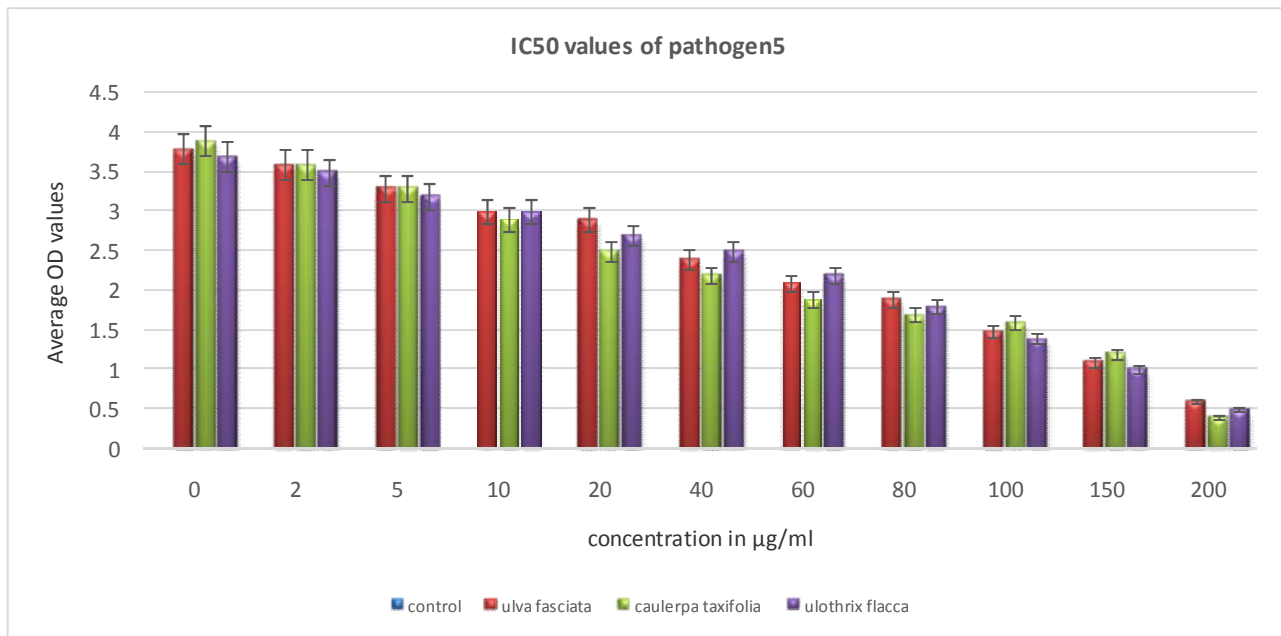
Graph 2. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-2



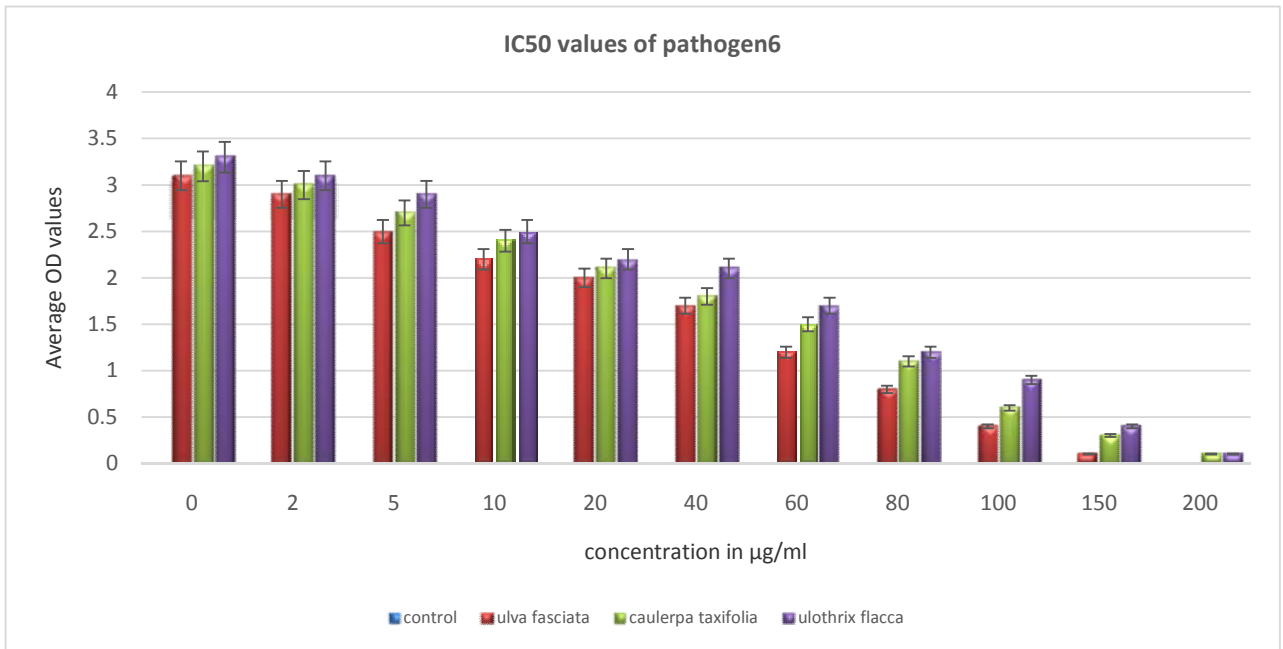
Graph 3. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-3



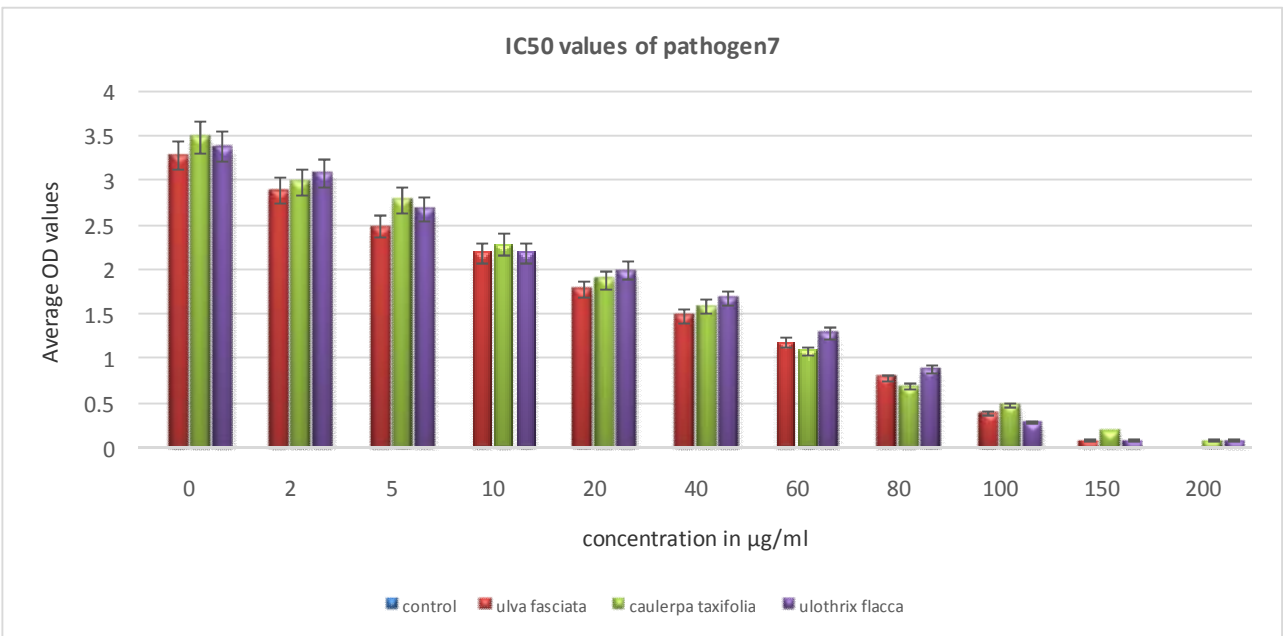
Graph 4. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-4



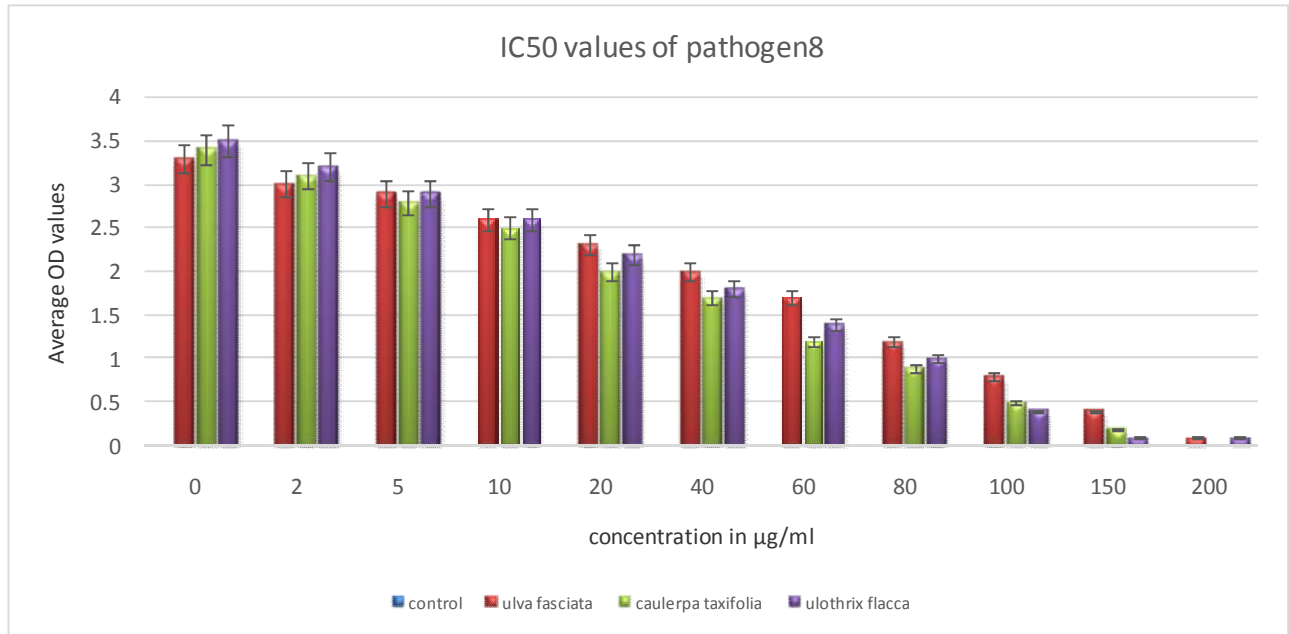
Graph 5. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-5



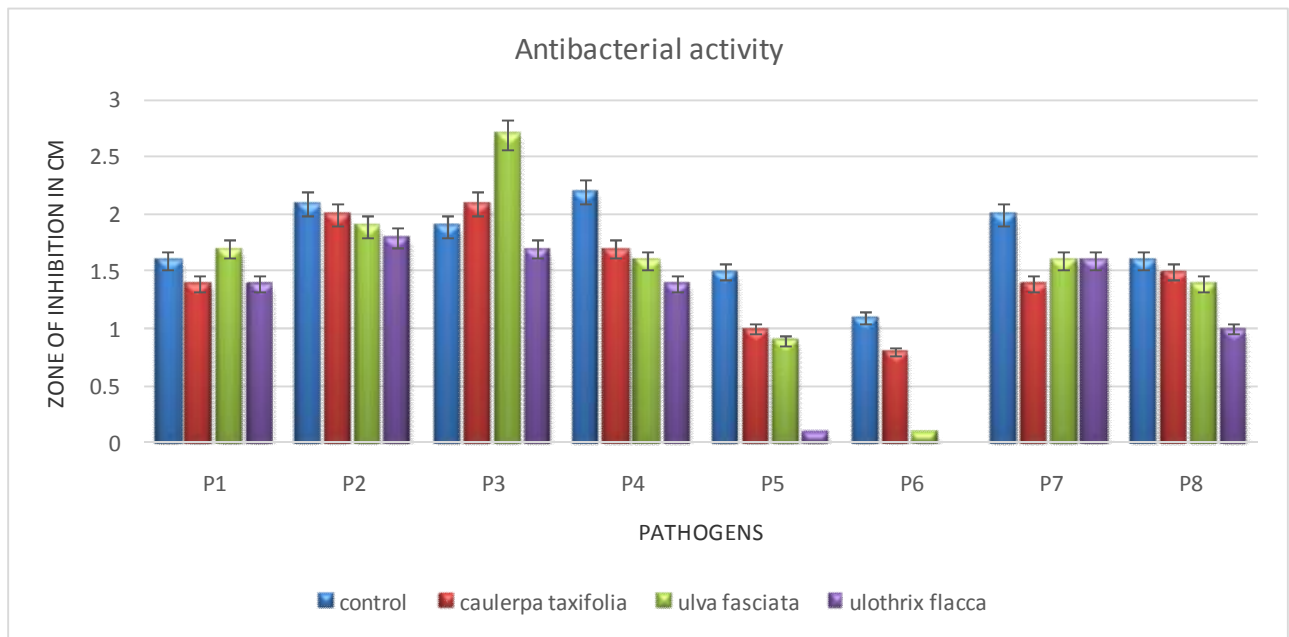
Graph 6. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-6



Graph 7. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-7



Graph 8. Estimation of MIC and IC₅₀ values of three seaweed extracts against pathogen-8



Graph 9. P1: *Mycobacterium spp.* P2: *Clostridium perfringens*, P3: *Listeria monocytogenes* P4: *Staphylococcus aureus* P5: *Vibriovulnificus* P6: *Plesiomonasshigelloides* P7: *Edwardsiellatarda* P8: *Delftiaacidovorans*.