

***In vitro* ANTI-OXIDANT AND ANTI-CANCER EFFICACY OF SILVER NANOPARTICLES SYNTHESIZED FROM THE SEAWEED *Syringodium isoetifolium* (D.) (1939) COLLECTED FROM THE PULICAT LAKE OF TAMIL NADU**

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Authors LJ and JG carried out the assays and experiments of the study. Author JJ designed the experimental setup for this study. Author MGR supervised the whole research work and corrected the manuscript draft. All authors read and approved the final manuscript.

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ABSTRACT

Seaweed is a natural, renewable and much unexplored marine resource, which are capable and reliable sources in the field of pharmaceuticals and drug discovery. The seaweed, *Syringodium isoetifolium* is our target plant for the study, which was collected from the Pulicat lake of Tamil Nadu. The obtained seaweeds were processed and the silver nanoparticles were synthesized from the aqueous extract of the plant material. The formation of silver nanoparticles was characterized using the UV visible spectroscopy, Scanning Electron Microscope (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). After the characteristic confirmation, the biosynthesized nanoparticles were evaluated for its antioxidant potential using various appropriate assays (DPPH, FRAP and ABTS), also the anti-proliferative efficacy of the same was determined using MTT assay against cancerous cell lines (COLO 320 and MCF-7).

Keywords: *Syringodium isoetifolium*; silver nanoparticles; anti-oxidant activity and anti-cancer activity.

1. INTRODUCTION

Our Earth comprises of 70% of water, with rich diversity of marine resources among which seaweed makes a vital contribution. As, they are the energy producers of the marine ecosystem, they uphold a

great position in the masses of ecological pyramid. Unlike other marine resources like fishes, oysters, crustaceans and molluscs, seaweed has less economic attraction and exploration among mankind. The carrageenan, alginate and agar products obtained from the seaweeds have wide applications in the

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fields of food, medicinal, and biotechnological industries [1].

Seaweeds are renewable energy resources which are comparatively difficult for accession, unlike the terrestrial and arboreal flora, therefore its phytochemical composition and their applications are to be deeply explored for its appropriate and efficient utilization. Deepak *et al.* [2] have reported the presence various bioactive metabolites in the seaweed extracts which has wide range of applications. Some of the bioactive metabolites present in the seaweed extracts like flavonoids, phenols, citric acid, ascorbic acid, polyphenolic, terpenes, alkaloids and reductase could act as reducing agents [3]. The seaweed extracts were found to possess numerous bioactivities such as anticoagulant [4], antioxidant [5], insecticidal [6], wound healing [7] anti-hypertensive [8] and anti-diabetic [9].

The bioactivity of seaweeds could be improved by deploying metal mediated synthesis of nanoparticles from them, whose activity must be more efficient and safe. Silver could be used in biological researches, as they were found to cause no hazard on utilization [10]. Pirtarighat *et al.* [11] have stated that the biologically synthesized silver nanoparticles could render more efficient and safer results in technical applications. Some of the metabolic processes in our body cause the release of free radicals which makes the cell unstable and thereby degrades it [12-15]. When such action was not counteracted by the body's natural defense mechanism, it may result in continuous generation of reactive oxygen species and that phenomenon is referred as oxidative stress [16,17] which may interrupt the cell functioning and have some adverse effects on the cellular targets such as lipid membranes, some proteins and the nucleic acids [18]. On long term conditions, this mechanism may lead to diabetes, rheumatism, arteriosclerosis, cancer, neural disorders and heart diseases [19]. Adding antioxidant supplements may help us to overcome the cell disruption caused due to ROS. Usually a diet comprising of more vegetables and fruits satisfies the antioxidant requirement of body but due to various factors and change in lifestyle, we are much dependent on antioxidant drugs for our wellbeing. This condition made researchers to screen more organic materials for their antioxidant properties.

The anti-oxidant properties of several bioactive active compounds were reported by many researchers [20-25]. More researches have been conducted on various plant materials to evaluate their antioxidant efficacies. The bioactive compounds present in algae could efficiently fight against the oxidative stress caused in

the body and there are many reports to support its possible usage as a natural antioxidant agent [26-31]. Some of the seaweeds like *Sargassum cristaefolium* [32]; *Sargassum incisifolium* [33]; *Desmarestia ligulata*, *Dictyota kunthii*, *Chondracanthus chamosoi*, *Laurencia chilensis* [34]; *Sargassum wightii* [2]; *Gracilaria edulis* [35] were screened for their antioxidant activity and their respective percentage radical scavenging activity were reported.

Cancer was reported to be the main cause of death all over the world and turned out to be a highly significant public health burden [36] and said to be the second leading cause of death in most of the developing countries [37]. A statistical study has estimated, about 9.6 million cancer-related deaths and 18 million new cancer cases in the year 2018 [38]. However, we are more advanced in cancer therapies, the usage of bioactive plant compounds for cancer prevention and treatment are still shown interest by the people than the synthetic chemical drugs [39,40]. Scientific report says that the naturally derived compounds contribute 60% for the drug discovery and were deployed as effective drugs to treat cancers [41]. Nanotechnology offers a wealth of tools to treat cancer by passing biological barriers to deliver therapeutic agents directly [42]. The toxicity of AgNPs is supported by the generation of double stranded DNA breaks along with chromosomal instability that drives the initiation of apoptotic execution [43,44]. This acting mechanism implies that AgNPs can be mutually associated with a great many DNA-targeting anticancer drugs. The silver nanoparticles proved unique anticancer activity against different types of cancer cells. Researches on nanotechnology cancer therapy extend beyond drug delivery into the creation of new therapeutics available only through use of nanomaterial properties. Although small compared to cell, nanoparticles are enough to encapsulate many small molecular compounds, which can be of multiple types. At the same time, the relatively large surface area of nanoparticle can be functionalized with ligands, including small molecule, DNA or RNA stand, peptides, aptamer or antibodies [45].

Enormous studies have been reported on the antioxidant and anti-cancerous properties in Ochrophyta (brown algae) and Rhodophyta (red algae) species [46-49]. The crude extracts of *Desmarestia ligulata* and *Dictyota dichotoma* were found to possess a great cytotoxic effect against the leukemia cell lines [47]. In addition to that, the bioactive compounds like phlorotannins and diterpenes obtained from algae of genus *Desmarestia* and *Dictyota* (Ochrophyta), where found to have better anti-oxidant and anti-carcinogenic effects [29].

Similar compounds isolated from *Laurencia undulate*, *Laurencia catarinensis* and *Laurencia microcladia* have been stated to act as anti-oxidant and anti- carcinogenic agents in treating skin, lung, prostate, and breast cancer lines [50-52]. Pulicat is the second largest lagoon in India and rich in aquatic faunal and seaweed diversity. *Ulva laculata*, *Ulva fasciata*, *Gracilaria textorii*, *Gracilaria verrucosa*, *Ectocarpus siliculosus*, *Chaetomorpha area*, *Enteromorpha sp.*, *Cladophora sp.*, *Syringodium sp.*, *Halophila ovalis*, *Kappaphycus alvarezii* and *Sargassum wightii* are some seaweed species recorded in Pulicat estuary [53-56]. Among them *Syringodium isoetifolium* is a type of sea grass which is a submerged hydrophyte, available only at specific seasons. Comparatively less works have been reported on the seagrass, therefore in this present study, the silver nanoparticles synthesized from the marine seagrass, *Syringodium isoetifolium* which was collected from the Pulicat lake of Tamil Nadu and were evaluated for their anti-oxidant and anti-proliferative efficacies.

2. MATERIALS AND METHODS

2.1 *Syringodium isoetifolium*

The marine seaweed *Syringodium isoetifolium* was collected from the mouth region of the Pulicat lake (13° 28'13.8" 80°18'30.8"), Thiruvallur district, Tamil Nadu during the month of October, 2019. The seaweed samples were brought to the laboratory and systematically identified. The seaweeds were well washed in tap water and then in distilled water to remove the dirt and soil particles. The cleansed plant material was shade dried in the laboratory for ten days at room conditions (30-35 °C temperature and 65-70% Relative humidity). The dried plant materials were finely clipped into smaller particles using a sharp scissors and then ground to powder using an electric blender, later stored in an airtight container for further processing.

2.2 Preparation of Algal Extract

About 10 g of seaweed powder added in 250 ml of distilled water in a conical flask which was stirred continuously for 25 min and then heated in water bath for 30 min at the temperature of 50-60 °C. The extract was then cooled and filtered using a whatman filter paper, where the obtained filtrate was transferred to a storage tube and then stored in a refrigerator [57].

2.3 Biosynthesis of Silver Nanoparticles

Silver nanoparticles were synthesized by following the method described by Abideen and Sankar [57], where 20 ml of pure algal extract was taken, to which

180 ml of 2mM silver nitrate solution was added and stirred well. Appearance of brown color after adding the silver nitrate solution indicates the formation of nanoparticle which was then monitored and confirmed using UV visible spectroscopy.

2.4 Characterization

Materialistic and characteristic natures of the synthesized nanoparticles were analyzed using various methods viz., UV-VIS Spectroscopy analysis, Fourier Transform Infrared spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). The UV visible absorption of spectra of silver nanoparticles or seaweeds extract was observed with a double beam spectroscopy between 380 nm to 480 nm. The different functional groups in nanoparticles were analyzed using Fourier Transform Infrared spectroscopy machine (model - FT/IR-6600typeA) and measurements were carried out using wavelengths of 349 cm to 7800 cm at infrared spectrum mode with 45°C angle of incidence.

2.5 Anti-oxidant Assays

2.5.1 DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

The DPPH assay was done by following the method described by Molyneux *et al.* [58]. About 3.7 ml of absolute methanol is taken in all test tubes and 3.8ml of absolute methanol was added to blank. 100 µl of BHT was added to tube marked as standard and 100 µl of respective samples were added to all other tubes marked as tests. 200 µl of DPPH reagent was added to all the test tubes including blank and all the test tubes were incubated at room temperature in dark condition for 30 min. The absorbance of all samples was read at 517 nm and the anti-oxidant activity was determined using the given formula:

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

2.6 ABTS Radical Scavenging Activity

The ABTS scavenging activity was determined by following the methods described by Re *et al.* [59]. The samples were diluted and taken in various concentrations of 20, 40, 60, 80, 100 µg/ml. About of 1.0 ml of diluted ABTS was added to 10 µl of different concentration of the sample and to 10 µl of methanol to make that as control. BHT was used as standard. The absorbance was read at 734 nm and the percentage inhibition was calculated using the prescribed formula.

ABTS scavenging activity (%) = $(A_0 - A_1) / A_0 \times 100$

Where,

A_0 is the absorbance of the control,

A_1 is the absorbance of the sample.

2.7 FRAP (Ferric Reducing Antioxidant Power) Assay

The FRAP assay was done to evaluate the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine complex and producing a colored ferrous tripyridyltriazine. This assay is more effective and can detect antioxidant potential of samples even at low as 0.2 mM Fe^{2+} equivalents. The FRAP assay was performed by following the methodology described by Benzie and Devaki [60]. 1 ml of water was added to each test tube named as the reagent blank, two standards and test samples which were then placed in the water bath at 37° C for 5 min. 100 μ l of standard, control or test sample was added to each tube and 100 μ l of water was added to the reagent blank tube and vortexed for 3 sec. further procedures were followed as mentioned and finally after the 4 min reaction, the contents were noted for absorbance in spectrophotometer at 593 nm against the blank. The FRAP value was calculated using the standard formula given.

FRAP value = Absorbance at 593 nm of test sample reaction mixture / Absorbance at 593 nm of Fe^{2+} STANDARD reaction mixture \times Fe^{2+} standard conc.

2.8 Anti-proliferative Assay

2.8.1 *In vitro* assay for determining the anti-cancer activity: (MTT assay)

The MTT assay for two cell lines (Colo 320 and MCF 7) was done by following the methods described by Mosmann [61]. Cells (1×10^5 /well) were plated in 24-well plates and incubated in 37° C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 h. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100 μ l/well (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-- tetrazolium bromide (MTT) was added and incubated for 4 h. After incubation, 1 ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration

required for a 50% inhibition (IC_{50}) was determined graphically. The percentage cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells \times 100

Graphs were plotted using the percentage cell viability rate 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.

3. RESULTS

3.1 Characterization of Synthesized Silver nanoparticles: UV-vis Spectroscopy Analysis

Uv visible spectrum was the primary characterization of synthesized nanoparticles and also used to monitor the synthesis and stability of AgNPs (Fig. 1). UV-vis spectrophotometer reading was observed in the range of 380 nm to 480 nm. The synthesized silver nanoparticles were monitored with the peak value of 410 nm.

3.2 FTIR Analysis

The synthesized silver nanoparticle biomolecules were confirmed using the FTIR spectral measurements. The different absorption of peak values obtained shows the presence of different functional groups of potential silver nanoparticles (Fig. 2).

3.3 SEM Analysis

Scanning electron microscopy was used for the morphological identification of size of the silver nanoparticles synthesized from *Syringodoium isoetifolium*. The nanoparticles synthesized were measured and found to vary in size from 42.15 and 54.35 nm in diameter (Fig. 3).

3.4 Antioxidant Assays

3.4.1 DPPH assay

The silver nanoparticles synthesized from the seaweed *S. isoetifolium* was evaluated for its radical scavenging activity using DPPH assay and found to possess 36.32% of scavenging activity at 100 μ g/ml concentrations, which was nearly half of the efficacy of the standard (BHT) used (Table 1 and Fig. 4).

3.5 ABTS Radical Scavenging Activity

The results of the ABTS assay performed using the silver nanoparticle, indicated the maximum activity of

11.31% at 100 $\mu\text{g/ml}$ and minimum of 4.42% activity at 20 $\mu\text{g/ml}$ concentrations (Table 2). The overall efficacy of synthesized nanoparticles was found to approximately similar to the standard used (Fig. 5).

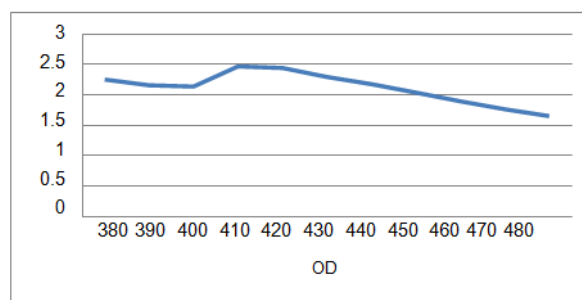


Fig. 1. UV spectroscopy analysis of silver nanoparticles

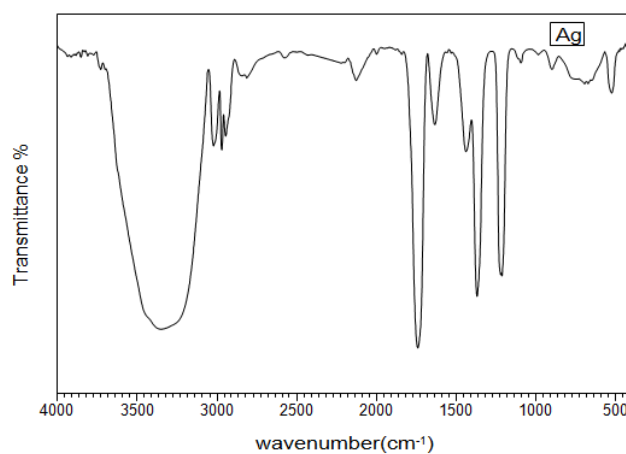


Fig. 2. FTIR analysis of synthesized silver nanoparticles

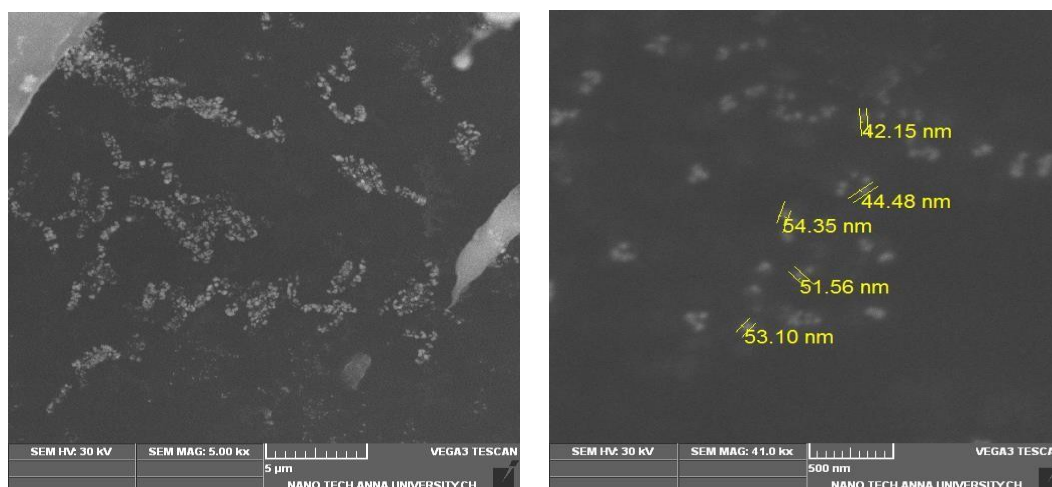


Fig. 3. SEM analyses of silver nanoparticles

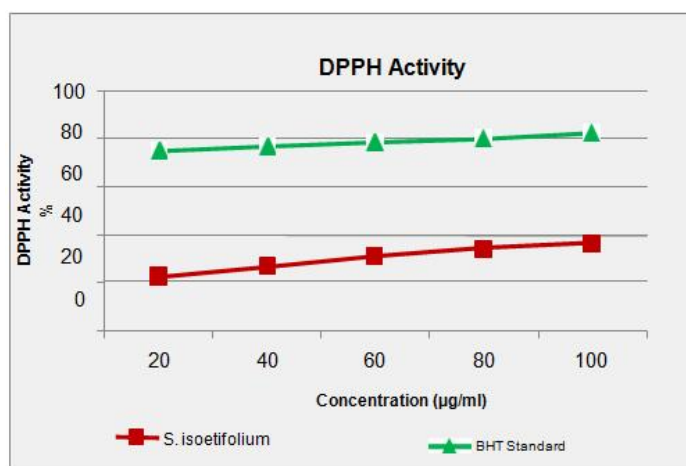


Fig. 4. Graph showing the DPPH radical scavenging activity of BHT standard and the synthesized silver nanoparticles

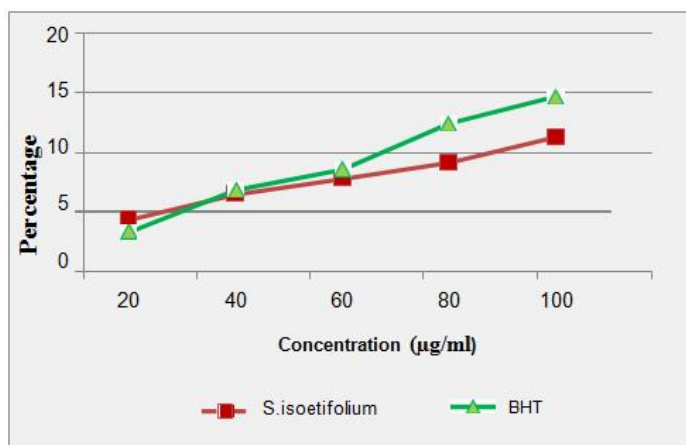


Fig. 5. Graph showing the ABTS radical scavenging activity of BHT standard and the synthesized silver nanoparticles

3.6 FRAP (Ferric Reducing Antioxidant Power) Radical Scavenging Activity

The results of the FRAP assay concluded that the efficiency of nanoparticles synthesized from *S. isoetifolium* was less than half of that of the ascorbic acid standard used (Table 3 and Fig. 6).

3.7 Anti-proliferative Assay

3.7.1 MTT assay on MCF 7 and COLO 320 cell lines

The cell viability was the parameter observed from the MTT assay, from which the results are to be

interpreted. The measured cell viability in the treated culture is the inverse proportionate amount of cells being killed by the synthesized silver nanoparticles. Therefore the minimal cell viability denotes the maximum cytotoxic activity. In that regard, the biosynthesized AgNPs were found to be highly efficient in controlling the COLO 320 cancer cell lines than the MCF 7 cells (Tables 4 and 5). About 75.92% of cellular inhibition was observed on MCF 7 cell lines and 92.50% of cellular inhibition was observed on COLO 320 cell lines, when exposed to AgNPs at 1000 µg/ml (Figs. 7 and 8). Figs. 9 and 10 shows the microscopic photographs of the treated and untreated cancerous cell lines.

Table 1. DPPH Radical scavenging activity of BHT standard and synthesized silver nanoparticles

S. No.	Concentration (µg/ml)	DPPH Activity %	
		STD	Test
1	20	75.30	22.33
2	40	77.20	26.40
3	60	78.71	30.80
4	80	80.02	34.10
5	100	82.32	36.32

Table 2. ABTS Radical scavenging activity of BHT standard and synthesized silver nanoparticles

S. No.	Concentration (µg/ml)	ABTS Activity %	
		STD	Test
1	20	3.39	4.42
2	40	6.94	6.55
3	60	8.64	7.86
4	80	12.46	9.18
5	100	14.73	11.31

Table 3. FRAP Radical scavenging activity of BHT standard and the synthesized silver nanoparticles

S. No.	Concentration (µg/ml)	FRAP Value (µmol/L)	
		STD	Test
1	20	376.66	214.44
2	40	584.44	282.22
3	60	783.33	357.77
4	80	1107.77	425.55
5	100	1308.88	515.55

Table 4. MCF 7 cell line anti-cancer effect of Si on MCF 7 cell line

S. No	Concentration (µg/ml)	Dilutions	Cell Viability (%)
1	1000	Neat	24.08
2	500	1:1	29.41
3	250	1:2	34.73
4	125	1:4	40.17
5	62.5	1:8	45.50
6	31.2	1:16	50.61
7	15.6	1:32	55.93
8	7.8	1:64	61.15
9	Cell control	-	100

Table 5. Anticancer effect of Si on COLO 320 cell line

S. No.	Concentration (µg/ml)	Dilutions	Cell Viability (%)
1	1000	Neat	7.50
2	500	1:1	13.47
3	250	1:2	19.72
4	125	1:4	26.11
5	62.5	1:8	32.36
6	31.2	1:16	38.61
7	15.6	1:32	44.86
8	7.8	1:64	51.11
9	Cell control	-	100

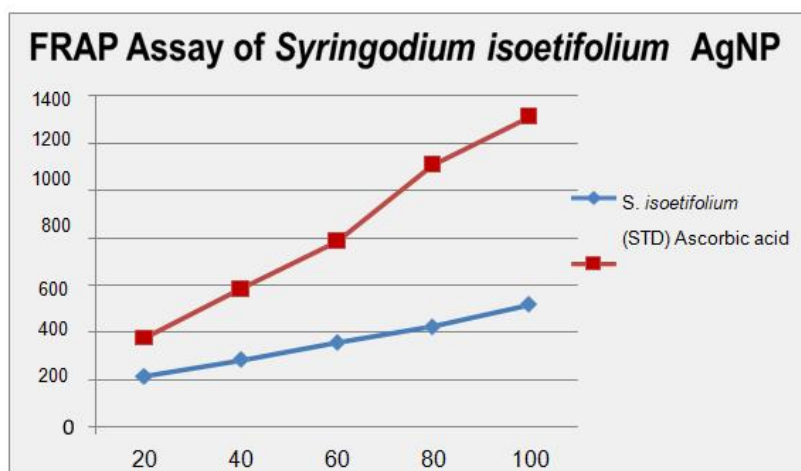


Fig. 6. Graph of FRAP Radical scavenging activity of BHT standard and synthesized silver nanoparticles

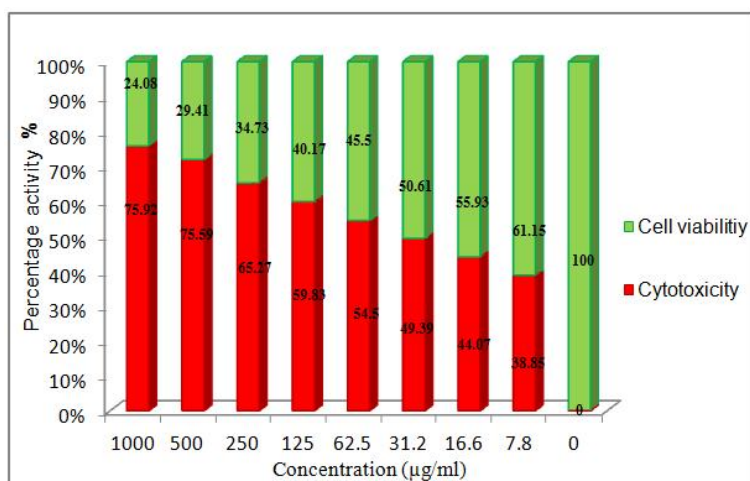


Fig. 7. Graph showing the anticancer effect of Si on MCF 7 cell line

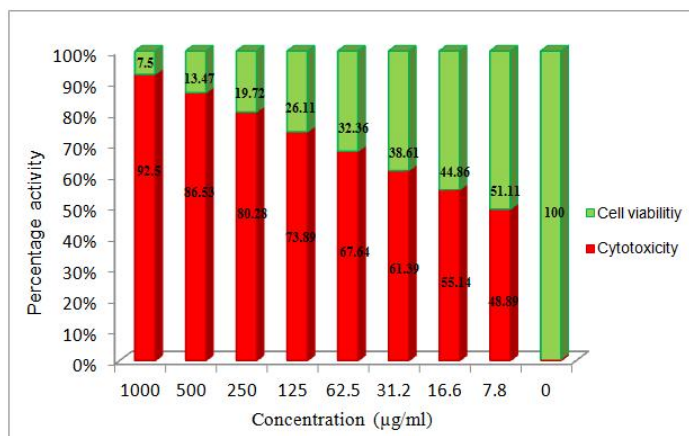


Fig. 8. Graph showing the anticancer effect of Si on COLO 320 cell line

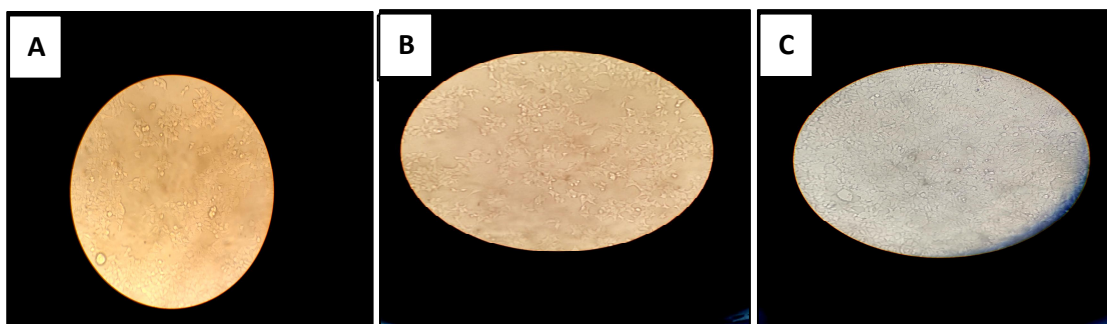


Fig. 9. Anticancer effect of AgNPs on MCF 7 cell line

A – 1000 µg/ml; B- 7.8 µg/ml; C – Normal control



Fig. 10. Anticancer effect of AgNPs on COLO 320 cell line

D – 1000 µg/ml; E- 7.8 µg/ml; F – Normal control

4. DISCUSSION

Man depends on various synthetic and commercially available elements to full fill his pharmaceutical requirements. Due to the resistance developed and side effects caused, people have started switching to natural and organic source of therapeutics, which is considered as safe. In that regard marine organisms, serves a great source of therapeutic bioactive compounds [29]. In recent times, people were observed to show higher interest on usage of phyto medicines for cancer treatment [62].

Seaweeds possess various and abundant secondary metabolites which have good radical scavenging activity and therefore could be utilized as antioxidant source [63]. *S. isoetifolium* has been reported to contain rich amount of phytochemical constituents like resin, glycosides, reducing sugars, saponins, acidic compounds, cardiac glycosides and alkaloids, one such phyto chemical among them could hold the antioxidant activity [64]. Vijayalingam and Rajesh [65] who has conducted a GC-MS study of the seaweed *S. isoetifolium*, has highlighted the pharmaceutical significances of that plant and the gas chromatography studies revealed the presence of decane, sucrose, phytol, hexadecanoic acid, 9,12,15-

octadecatrienoic acid (Z,Z,Z)- (fatty acid derivative), Triethylene glycol monododecyl ether and 2-hydroxy-1 (hydroxymethyl) ethyl ester in that. On referring with the phytochemical database [66], we could arrive with a clue that the presence of n-Hexadecanoic acid (a diterpene alcohol) and Linoelaidic acid (a linoleic acid isomer) would be active compound for anti-oxidant and anti-cancer activities.

In this study we have critically evaluated the antioxidant efficacy of AgNPs synthesized from *S. isoetifolium* using three different methods viz., DPPH, ABTS and FRAP assays. Among that The DPPH and ABTS assays are reported as widely accepted methods for assessing antioxidant efficacies of biological products through spectrophotometric estimations of their stable colored radicals [67]. When evaluated for DPPH scavenging assay, the reaction of the biosynthesized AgNPs of *S. isoetifolium* with the violet colored (DPPH added) solution was observed. The plant material of *S. isoetifolium* exposed would have rendered ions to break the free radical chain and there by on incubation results in bleaching of the natural color formed due to DPPH addition. In our study, the silver nanoparticles of *S. isoetifolium* exhibits 36.32 % of DPPH scavenging activity at 100

µg/ml which was lesser than half of that of the reaction caused by BHT (Butylated hydroxytoluene) (Fig. 1). The scavenging activity of the nanoparticles on ABTS radicals were quantified by measuring the absorbance at 734 nm and was found to be 11.31 % at 100 µg/ml concentration. The observed values are interpreted to be slightly lesser than the control (BHT) used (Fig. 2). However, the plant based antioxidant products are natural and has longer durability and stability.

In the ferric reducing antioxidant power (FRAP) assay performed, instead of the transfer of hydrogen atoms the electron transfer determines the antioxidant potential of the material tested [68]. The reducing power of AgNPs converts the ferri cyanide Fe_3^{+} to ferrocyanide Fe_2^{+} by donating an electron, which is marked by a color change. FRAP is a simple and efficient technique to determine the antioxidant property of the test substance based on the iron molecule reduction capability and to be conducted in acidic medium to maintain iron solubility [69]. The results of our study indicated the moderate FRAP values (515.55 µmol/l at 100 µg/ml), when compared to the standard ascorbic acid used (Fig. 3). At 20 µg/ml, the FRAP value determining the Fe ion reduction property was found to be 214.44 µmol/l.

In the treatment of cancer surgical or radio therapies are found to effective only if the disease has been detected earlier, but for most of cancers which were diagnosed at later stages (when already metastasized), in that cases chemotherapy renders effective results [70]. Utilization of seaweed components for *in-vitro* cancer cell treatments are reported by various researchers and also found to be effective [71,33,72, 73,35]. The synthesized AgNPs were tested against the cell lines (MCF 7 AND COLO 320) and found to be efficient in making them inactive. The results of the anti-proliferative studies conducted by MTT assay on MCF 7 cell lines indicated the maximum cytotoxic activity (67.82 %) and minimum cell viability (32.18 %) at 1000µg/ml of the biosynthesized AgNPs. The cell viability rate varies with the concentration of silver nanoparticles exposed and maximum cell viability (67.36 %) was observed at 7.8 µg/ml of silver nanoparticles synthesized from *S. isoetifolium*. Approximately equal rates of cell viability and cytotoxic have been observed at 62.5 µg/ml of the test sample.

Similarly when evaluated with COLO 320 cell line higher cytotoxicity (92.50%) was observed when exposed to 1000 µg/ml of silver nanoparticles. It was found that 15.6 µg/ml of the nanoparticles were required to make 51.67 % of the cancer cells inactive.

When the cancer cells were exposed to the lower concentration (7.8 µg/ml), 45.56 % of cytotoxicity was noted. In overall, the AgNps synthesized from *S. isoetifolium* was more efficient in controlling COLO 320 cell lines than MCF 7. Angel [74] has evaluated maximum cytotoxicity of 88.46% at 1000 µg/ml concentrations and minimum of 59.68% at 62.5 µg/ml of methanolic extract of *S. isoetifolium*, whereas in our study we have synthesized AgNPs using the aqueous extracts of the same plant and evaluated.

5. CONCLUSION

The silver nanoparticles of the seaweed *S. isoetifolium* synthesized were found to be cost effective, reliable and eco-friendly. On evaluation, the synthesized nanoparticles were identified as potential antioxidant and anticancer agents which could be used for therapeutic purposes as an alternate to other chemical drugs, which pose the fear of causing side effects and developing resistance. There by exploring the detailed bioactivity of naturally available products like seaweeds may pave way for drug discovery for various ailments.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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