



## ***In vitro* antioxidant and antiproliferative activity of crude fucoidan from *Sargassum* species**

**Ashra Sindhikkaa Mohammed Rafi and Veerabhuvaneshwari Veerichetty**

Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, Tamil Nadu, India-641049.

[ashrasindhikkaa.18mbt@kct.ac.in](mailto:ashrasindhikkaa.18mbt@kct.ac.in)

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### **Abstract**

Fucoidans are sulfated heteropolysaccharides majorly composed of L-fucose commonly present in brown algae and some marine invertebrates. Cancer is the second leading cause of death worldwide and the current treatment with chemotherapeutic drugs has a major disadvantage of causing various toxic side effects. Fucoidan was reported to have various biological activities including anticancer activity. In this study, polysaccharide fraction was extracted from the brown algae *Sargassum* species followed by its fractionation using anion exchange chromatography. FTIR spectroscopy was used for the characterization of fucoidan in comparison with the commercially available fucoidan. The antioxidant activity of crude fucoidan was determined using DPPH, hydroxyl radical and superoxide anion radical scavenging assay. MTT assay was used to determine the cell reducing capacity of the crude fucoidan against cervical cancer cells (HeLa cells) with paclitaxel as the standard chemotherapeutic drug control. The IC<sub>50</sub> value of crude fucoidan in HeLa and HEK cells were found to be 24 & 36 µg/ml and IC<sub>50</sub> of Paclitaxel was 30 & 20 nM. Fucoidan was reported to get accumulated in the kidneys due to less bioavailability. Hence, further studies has to be done to increase the bioavailability of fucoidan to enhance the cell reducing capacity.

**Keywords:** Anion exchange chromatography, crude fucoidan, HeLa cells, HEK cells, *Sargassum sp*

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### **1. INTRODUCTION**

Fucoidan is a heteropolysaccharide that is majorly composed of fucose and sulfate esters. It also comprises of other monosaccharides like galactose, glucose, mannose, rhamnose, arabinose and xylose, uronic acids, proteins and acetyl groups (Li, Lu, Wei, & Zhao, 2008) & (Wang, et al., 2019). Although its structure and composition varies depending on the species (Cumashi, et al., 2007), extraction procedures (Wang & Chen, 2016) and season of collection (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010) which in turn influences its activities. Brown marine algae contains polysaccharides in large amounts in their cell wall which are mostly sulfated polysaccharides. Thus, the sulfated polysaccharide fucoidans are commonly present in marine brown algae and some marine invertebrates and not in any other seaweeds or plants. Algal edible polysaccharides have gained huge interest in science due to their diversified biological activities (Manojkumar.K & Seema, 2013). These biological activities include antitumor, antimicrobial, anti-inflammatory, anticoagulant, antioxidant, immunoregulatory and antithrombotic activities (Chizhov, et al., 1999) & (Sinurat, Peranginangin, & Saepudin, 2016). Fucoidan was extensively used as a traditional medicine in China for over 2000 years especially from brown algae *Sargassum* species to treat various conditions like thyroid (goiter), edema, renal issues, tumors, ulcer, scrofula, testicular pain, asthma, scabies, eczema, swelling and psoriasis. The fucoidan was first extracted and identified in the year 1913 from various species of brown algae such as *Fucus vesiculosus*, *Ascophyllum nodosum* and *Laminaria digitata*. Further it is also identified to be a negatively charged polysaccharide which is hygroscopic and soluble in water and acids (Luthuli, et al., 2019). It was reported that, fucoidan with high sulfate content and low molecular weight possessed high degree of antitumor and other biological activities. Whereas this is vice versa in few rare cases of fucoidan depending on the species used for extraction (Anastyuk, et al., 2012).

Cancer is the second leading cause of death worldwide after cardiovascular disorder. Over 277 different types of cancers had been reported so far (Hassanpour & Dehghani, 2017). Cancer is a condition where the cells in the body begin to grow and reproduce in an uncontrollable manner which confers various threat to the host. This is caused by genetic mutation due to external factors which in turn activates or inactivates specific genes which alters the regulation of mechanism in the human body (Danaei, et al., 2005). These cells then invade and destroy healthy tissues, including organs and this condition is called metastasis. According to GLOBOCAN report of 2018, around 18.1 million new incidence of cancer and 9.6 million mortalities have been recorded. Further it predicts that incidence and mortality can

be increased to over 15.1 million and 12 million by the year 2020. The report also states that highest number of mortality due to cancer is prevalent in the Asian region (57.3%) followed by Europe (20.3%), America (14.4%) and Africa (7.3 %). Also among the 57.3% of mortality in Asian region, 7.8% is accounted by India (Kishore & Kiran, 2019). In India, the cancer mortality rate has been doubled from 1990 to 2016 (Smith & Mallath, 2019). According to World Health Organisation (WHO) report of India, cancer accounted for 7.6 million deaths in the year 2008 and expected to be over 13.1 million by the year 2030. Similarly, the world's population by the year 2020 is estimated to be around 7.5 billion and it is predicted that, about 15 million new incidence of cancers is expected to be diagnosed followed by the mortality of 12 million patients among them (Bray & Møller, 2006).

The tumor can be treated effectively when diagnosed at the early stage. The common and standard procedures to treat any cancer conditions are surgery, radiotherapy and chemotherapy. The type of treatment depends on various factors but majorly on the size and type of the cancer. Surgery is the case where the affected area is removed from the host thereby preventing further development or invasion for metastasis. Radiation is the treatment employing high energy X-rays to reduce or shrink the spread of cancer cells. It is applied along with either surgery or chemotherapy for increased efficiency and it is not much effective when employed alone. It is less dangerous since it only targets the tumor region (Huang, Ju, Chang, Muralidhar Reddy, & Velmurugan, 2017). Thus surgery and radiotherapy was used worldwide to treat cancer till 1960s when it was realized that it is not as effective as expected since the cancer prolonged to develop due to metastasis. Followed by this in the 1960s and 1970s, combination chemotherapy cured Hodgkin's disease and acute leukemia and after several researches, chemotherapeutic drugs came into use in the 20<sup>th</sup> century providing promising results in the treatment of cancer. But later on it was realized that use of chemotherapeutic drugs to treat cancer imposed various toxic side effects on the patient which further degraded their health. The signs of side effects may be immediate or appeared later. However, even today chemotherapeutic drugs are used to treat cancer which have a major disadvantage of causing various toxic effects to the host like serious side effects, drug resistance and also targeting the normal cells and tissues due to the general non-specific deregulation processes (DeVita & Chu, 2008).

Cervical cancer is found to be the fourth most common and mortality causing cancer among women. Further the incident rate of cervical cancer was recorded to be rapidly increasing among young women (Kong, Zong, Yang, Wu, & Xiang, 2019). Like almost all kind of cancers, cervical cancer can also be treated or even cured effectively on early diagnosis. Also it is found to have long pre-invasive period and can be found only on the surface layer of the cervix which is a major advantage of early diagnosis and treatment which reduces the mortality rate. The most commonly used treatment for cervical cancers after various evolution is radiotherapy combined with surgery or concurrent chemoradiation wherein radiotherapy performed along with the uptake of chemotherapeutics. Chemotherapy was also used to treat cured and invaded back cervical cancer. Both surgery along with radiation and chemoradiation causes various side effects among which some are toxic (Šarenac & Mikov, 2019).

The toxic effects of chemotherapy lead to the extensive research on finding a cure to cancer that is less or no toxic and specific and effective to treat the tumor. Bioactive compounds from natural resources has proven to be non-toxic through various studies. Fucoidan is a heteropolysaccharide which is already reported to be a potential inhibitor of cancer also it had proven to reduce the toxic effects imposed by chemotherapeutic drugs. But the bioavailability of fucoidan was found to be less. Only a small amount of fucoidans administered into the host had crossed the intestinal walls whereas almost all fucoidan molecules tends to accumulate in kidneys which was then later excreted in urine. In this way the potential of fucoidan is decreased in treating cancer cells due to less bioavailability (Chen, et al., 2017) & (Udani, 2012). Further fucoidans are reported to be toxic against human embryonic kidney (HEK) cells which may be responsible for the accumulation of fucoidan in kidneys (Mak, et al., 2014). Further, low molecular weight fucoidan (LMWF) was found to have less sulfate ester group which in turn has proven to be more effective than high molecular weight fucoidan (HMWF) with high sulfate ester groups. Hence, it is highly beneficial to concentrate LMWF for its bioactivities (Saepudin, Qosthalani, & Sinurat, 2018). Seaweeds are already being used industrially in various fields. Similarly, not only clinical but various fields have been opened up for fucoidan owing to its health benefits. Fucose rich sulfated polysaccharides isolated from brown seaweed shows promising results for potential opportunities in nutraceuticals, pharmaceuticals, cosmeceuticals and functional foods (Cunha & Grenha, 2016). However, appropriate processing technologies has to be incorporated to obtain its functional properties along with enhanced bioavailability (Wijesinghe & Jeon, 2012).

.In this study, fucoidan was extracted (Kordjazi M, Etemadian, Shabanpour B, & Pourashouri, 2019). from the brown marine algae *Sargassum* species followed by fractionation using anion exchange chromatography (Koyanagi,

Tanigawa, Nakagawa, Soeda, & Shimeno, 2003), (Marudhupandi, et al., 2014), (Na, et al., 2008) & (WJ Kim, et al., 2007). The positive fractions were then spray dried and the powdered sample is characterized using FTIR spectroscopy in the wavelength range of 4000-400  $\text{cm}^{-1}$  (Liu, Liu, Wei, Sun, & Wang, 2016) & (Shanthi, Eluvakkal, & Arunkumar, 2014).

The anti-oxidant activity of the obtained crude fucoidan is determined using free radical scavenging assay (DPPH) (Lim, et al., 2014), hydroxyl radical scavenging assay (Lim, et al., 2014) and superoxide anion radical scavenging assay (Zhao, et al., 2018). The cell reducing capacity of the crude fucoidan against cervical cancer cells (HeLa) and human embryonic kidney (HEK) cells were determined using MTT (3- (4, 5- dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide) assay (Vaseghi, Sharifi, Dana, Ghasemi, & Yegdaneh, 2018), (Karthick, et al., 2019) & (Narayani, Saravanan, Ravindran, Ramasamy, & Chitra, 2019).

## 2. MATERIALS AND METHODS

### 2.1 Algae collection and sample preparation

The brown seaweed *Sargassum* species was collected from the coastal areas of Tuticorin. The collected marine algae was dried over a month till no moisture content is left behind. The dried sample was then homogenized completely.

### 2.2 Extraction of polysaccharide fraction

10 gram of powdered sample was dissolved in 500 ml of distilled water and boiled at 85°C for around 2 hours. The solution was then cooled down to room temperature and filtered. The pH of the filtrate was adjusted to 7 to increase the yield of extraction. 4% trichloroacetic acid (TCA) was added to the solution and incubated overnight at 4°C. The precipitated proteins were then removed through centrifugation and the resulting supernatant was collected. Thrice the volume of absolute ethanol (100%) was added to the supernatant and incubated overnight at 4°C. The precipitated polysaccharides containing fucoidan was collected through centrifugation. The pellet formed was completely dried in the hot air oven or through air drying (Kordjazi M, Etemadian, Shabanpour B, & Pourashouri, 2019).

### 2.3 Fractionation of crude fucoidan using anion exchange chromatography

Sulfated polysaccharides are negatively charged particles hence for the fractionation of fucoidan, anion exchange chromatography was used wherein the matrix is composed of negatively charged particles. The column used here in the separation of fucoidan from other impurities was tightly packed DEAE Sepharose. The mobile phase used was 1M Tris HCl buffer with the pH in the range of 6.7-7. The column was equilibrated with the freshly prepared 1M Tris HCl buffer. The extracted sample was dissolved in the mobile phase Tris HCl buffer and loaded onto the column. Followed by this, the sample was eluted using gradient elution with NaCl in the concentrations range of 0.5M-2.5M. The eluted samples were then collected in 50 fractions and each fraction was tested for the presence of sugars by anthrone's test (Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003), (Marudhupandi, et al., 2014), (Na, et al., 2008) & (WJ Kim, et al., 2007). Anthrone's test was performed by adding the anthrone reagent to each fraction in the ratio of 2:1. The bluish green colour was observed and further the absorbance value of the fractions were measured at 630nm after the incubation of 15 minutes at room temperature. Galactose was used as the standard in the concentration range of 20-100 $\mu\text{g/ml}$ . The obtained absorbance value was plotted in a graph and compared with the standard values obtained for quantification. The positive fractions were then pooled together (Morán-Santibañez, et al., 2016) & (Katoch & Katoch, 2011).

### 2.4 Nano spray drying

The extracted sample was completely purified using anion exchange chromatography. The collected positive fractions were then nano spray dried to get powdered fucoidan from the purified liquid sample and stored at 4°C until use.

### 2.5 Characterization of fucoidan by FTIR spectroscopy

The nano spray dried fucoidan was analyzed using Fourier transform infrared spectroscopy (Shimadzu, Japan) with wavelength in the range of 4000-400  $\text{cm}^{-1}$ . The resulting spectra directly corresponds to the functional groups present in the structures of given fucoidan sample.

## 2.6 Antioxidant activity

### 2.6.1 Free radical scavenging activity

The free radical scavenging activity of the crude fucoidan was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals. The reaction mixture consists of 0.15 mM DPPH in methanol (Lim, et al., 2014). 1 ml of fucoidan sample in varying concentrations (20-100 µg/ml) was added to 2.9 ml of DPPH. The reaction mixture was then incubated in the dark for 30 minutes. Followed by this the absorbance was measured at 517 nm. Ascorbic acid was used as the standard with same concentrations as the sample. Both the standard and sample mixtures were prepared in triplicates. The free radical scavenging activity of crude fucoidan and ascorbic acid can be determined using the formula given below.

$$\text{Free radical scavenging activity (\%)} = [(A_1 - A_2) / A_1] \times 100$$

Where  $A_1$  is the absorbance of DPPH blank and  $A_2$  is the absorbance of the DPPH in presence of sample.

### 2.6.2 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the fucoidan sample was determined using Fenton's reaction. The Fenton's reagent can be prepared by adding ferrous sulphate ( $\text{FeSO}_4$ ) to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) which then generates hydroxyl radical (Lim, et al., 2014). 0.5 ml of 9 mM ferrous sulphate solution was added to 1.0 ml of 8.8 mM hydrogen peroxide solution. To the prepared Fenton's reagent varying concentrations (200-1000 µg/ml) of fucoidan was added followed by the addition of 0.2 ml of 9 mM salicylic acid solution. Salicylic acid was added to one set and another set of the same reaction mixtures were prepared without salicylic acid. The reaction mixtures were then incubated at 37°C for 1 hour and their absorbance were measured at 510 nm. The hydroxyl radical scavenging activity of the fucoidan sample was then compared to the standard ascorbic acid measured at the same concentrations (200-1000 µg/ml). Both the sample and the standard reaction mixtures were prepared in triplicates. The hydroxyl radical scavenging activity in percentage was determined using the formula given below.

$$\text{Hydroxyl radical scavenging rate (\%)} = [1 - \{(A_1 - A_2) / A_0\}] \times 100$$

Where,  $A_1$  is the absorbance of the reaction mixture with salicylic acid,  $A_2$  is the absorbance of the reaction mixture without salicylic acid and  $A_0$  is the absorbance value of control.

### 2.6.3 Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was determined using pyrogallol auto-oxidation method. The reaction mixture consists of 50 mM Tris-HCl buffer with pH 8.2 and 6 mM pyrogallol contained in 10 mM HCl (Haitian Zhao, 2012). 0.2 ml of varying concentrations of fucoidan were incubated with 5.7 ml of Tris-HCl buffer at 25°C for 10 minutes. Later 0.1 ml of pyrogallol solution was added onto the mixture and incubated for another 5 minutes. Followed by that, the absorbance was measured at 320 nm. The superoxide anion radical scavenging activity of the fucoidan sample was then compared to the standard ascorbic acid measured at the same concentrations (200-1000 µg/ml). Both the sample and the standard reaction mixtures were prepared in triplicates. The superoxide anion radical scavenging activity in percentage is determined using the formula given below.

$$\text{Superoxide anion radical scavenging rate (\%)} = [1 - \{(A_1 - A_2) / A_0\}] \times 100$$

Where,  $A_0$  is the auto-oxidation rate of pyrogallol (the change in absorbance of the solution containing pyrogallol in Tris-HCl at 0<sup>th</sup> minute and after 15 minutes),  $A_1$  is the oxidation rate of pyrogallol in presence of each sample concentration (the change in absorbance of the solution mixture containing pyrogallol in Tris-HCl along with samples at 0<sup>th</sup> minute and after 15 minutes) and  $A_2$  is the absorbance of the sample blank of each varying concentration.

### 2.6.4 Cell reducing capacity (cytotoxicity) assay

The antiproliferative capacity of crude fucoidan against cervical cancer cell HeLa and non-cancer cell line human embryonic kidney (HEK) cells was determined using MTT assay. Further, Chemotherapeutic drug paclitaxel was used as standard drug control. The cervical cancer cell line (HeLa cells) and human embryonic kidney (HEK) cells were purchased from the National Centre for Cell Sciences (NCCS) located in Pune, India. The cell lines obtained were then cultured and maintained in Dulbecco's modified eagles medium (DMEM) containing 2 mM of 1-glutamine, balanced salt solution (BSS) that was adjusted to have 1.5 g/L of  $\text{Na}_2\text{CO}_3$ , 1 mM of sodium pyruvate, 1.5 g/L of glucose, 0.1 mM

of non-essential aminoacid, 10 mM of HEPES (4-2-hydroxyethyl)-1-piperazineethane sulfonic acid) and 10% FBS (fetal bovine serum). Also the quantity of antibiotics, penicillin (100 units/ml) and streptomycin (100µg/ml) added to the medium was in the range of 1ml/L. The cells cultured were then maintained at 37°C in the presence of 5% CO<sub>2</sub> through incubating in a humidified CO<sub>2</sub> incubator (Ciapetti, Cmni, & Pratelli, 1992) & (Kanipandian, Kannan, Ramesh, Subramanian, & Thirumurugan, 2014).

The HeLa and HEK cells were cultured in a 96 well plate in the concentration of 1x10<sup>4</sup> cells/well for 48 hours which gives around 75% confluence. Followed by this, the utilized medium was replaced by the fresh medium and the cells were again incubated for another 48 hours in the fresh medium containing supplemented nutrients required for their growth and survival. After 48 hours, the utilized medium was removed from the culture and to the cultured cells 100 µl of the MTT (3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide) solution was added onto the each well of 96 well plate and incubated for 4 hours at 37°C. Followed by the incubation, the supernatant was removed and to the pellet 50 µl of DMSO (Dimethyl sulfoxide) solution was added onto the each well containing the cells and incubated for another 10 minutes to solubilize the formazan crystals formed upon cell reducing capacity of the samples against cancer cells. Finally the optical density of cells in each well was measured at 620 nm using ELISA plate reader. From the absorbance value obtained, the percentage of viability of the cell lines can be calculated using the formula given below.

$$\% \text{ of viability} = (A_1/A_0) \times 100$$

Where, A<sub>1</sub> is the absorbance of the experimented sample and A<sub>0</sub> is the absorbance of the control used.

The percentage of viability corresponds to the cell reducing capacity of the crude fucoidan sample.

### 3. RESULTS AND DISCUSSION

#### 3.1 Extraction of crude fucoidan

The crude fucoidan extracted and dried were by around 28mg. Initially 10g of the powdered seaweed was taken for extraction. The percentage yield for the fucoidan extraction process was calculated using the formula given below.

$$\text{Yield (\%)} = (W_1 / W_0) \times 100$$

Where, W<sub>1</sub> is the final weight of the extracted fucoidan and W<sub>0</sub> is the initial seaweed provided [26].The yield (%) was calculated to be 0.25%.

#### 3.2 Fractionation using anion exchange chromatography

The extracted fucoidan was purified using anion exchange chromatography in DEAE Sepharose column through gradient elution with NaCl (0.5M-2.5M). The fractions collected were then tested using anthrone's reagent to determine the sugar positive fractions. Further the fractions were quantified by comparing with the standard galactose. From the graph in the Figure 1 it was observed that, the absorbance value was increased from the fractions 20 to 40 which was eluted by the gradients 1.5 & 2M NaCl. Hence the crude fucoidan was purified by eluting with the 1.5 & 2M NaCl and pooled together. The graph in the Figure 2 signifies the standard graph of galactose and Figure 3 shows the corresponding concentrations of fucoidan in each fractions extrapolated by galactose equivalence.

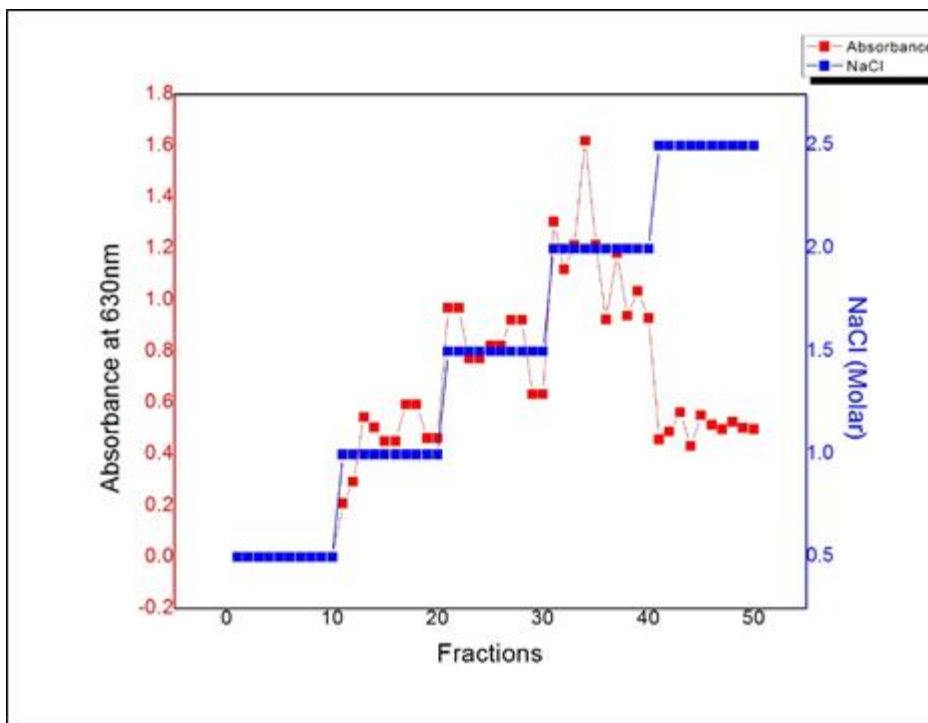


Figure 1. The absorbance value obtained for each fraction eluted in anion exchange chromatography was plotted against the fraction number (0-50) and the corresponding concentration of sodium chloride (0.5-2.5 M) used for the elution. Increase in absorbance was observed between the peaks 20 and 40 corresponding to the presence of polysaccharide fractions.

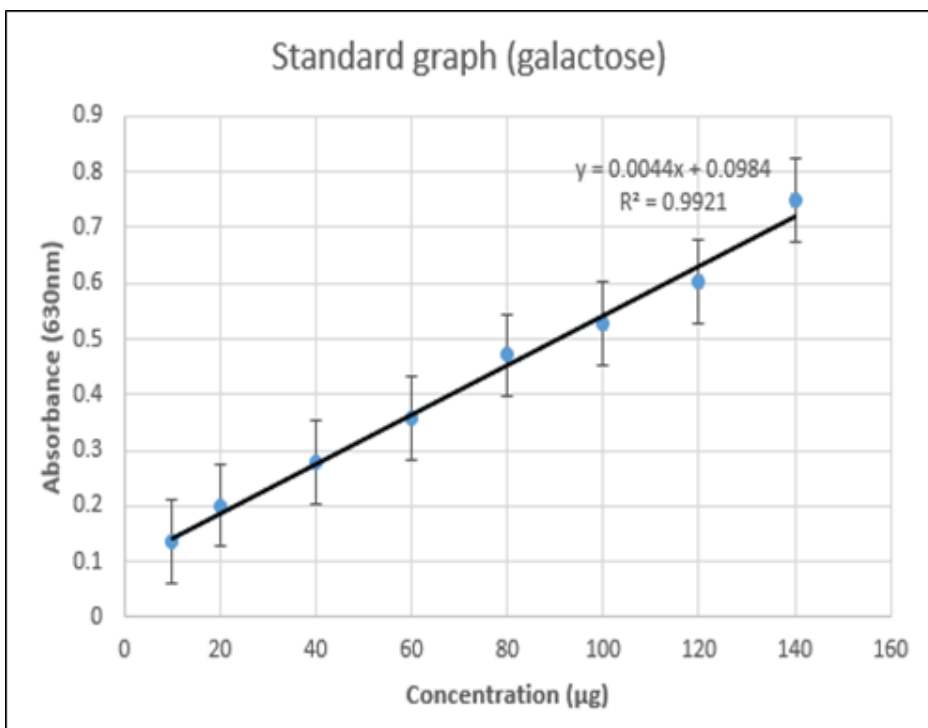
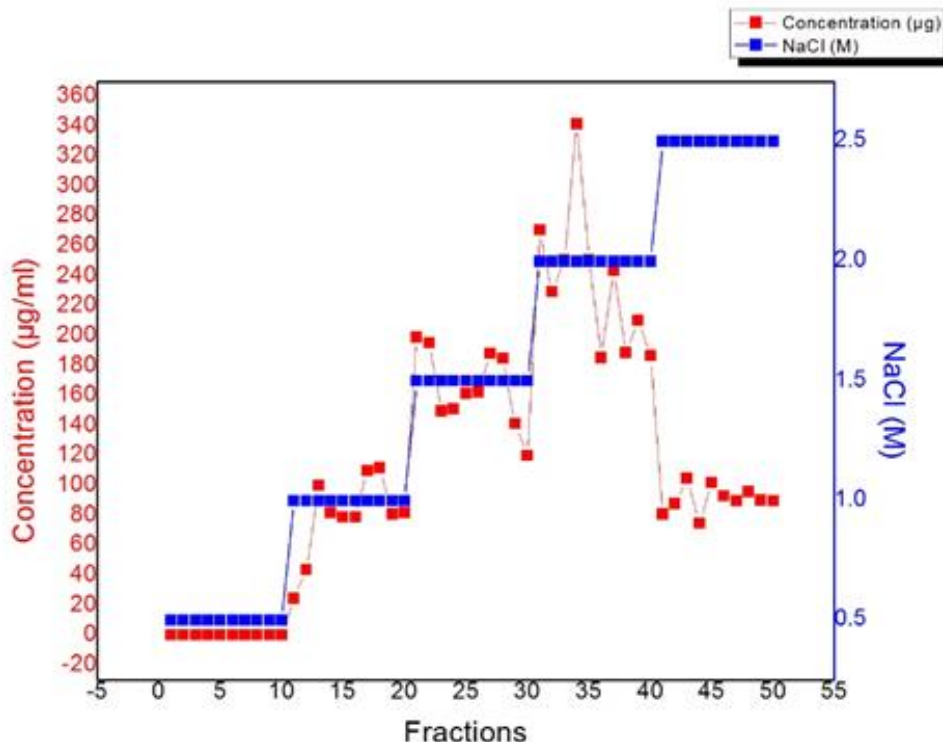


Figure 2. Standard graph of galactose equivalence.



**Figure 3.** The absorbance obtained for each fraction was extrapolated with the concentrations using the standard graph of galactose equivalence.

### 3.3 Nano spray drying

The pooled fucoidan fractions were nano spray dried to collect the powdered crude fucoidan. The powdered sample was fine particles and white in colour. The nano spray dried crude fucoidan was around 30 grams and stored at 4°C until use.

### 3.4 Characterization of fucoidan by FTIR spectroscopy

The nano spray dried fucoidan was analyzed using Fourier transform infrared spectroscopy (Shimadzu, Japan) with wavelength in the range of 4000-400  $\text{cm}^{-1}$ . From the spectra in Figure 4, it was observed that the bands at 3186.40 and 3101.54  $\text{cm}^{-1}$  corresponds to the presence of O-H stretching vibration. Similarly the band at 2974.23  $\text{cm}^{-1}$  corresponds to the C-H stretching which signifies the presence of pyranoid ring and C<sub>6</sub> of fucose. The bands at 1392.61 and 1041.56  $\text{cm}^{-1}$  corresponds to the S=O stretching signifying the sulfate groups connected to the sugars. The peak at 906.54  $\text{cm}^{-1}$  signifies the presence of bending vibration of C-O-S bonds. Further, the peak at 1138  $\text{cm}^{-1}$  corresponds to the glycosidic bond C-O-C. The band at 1296.16  $\text{cm}^{-1}$  corresponds to the C-O stretching signifying the presence of aromatic esters. Hence from the spectra it was characterized that the extracted and purified sample contains the crude fucoidan with presence of other few compounds which is responsible for other peaks (Isnansetyo, Lutfia, Nursid, Trijoko, & Susidarti, 2017) & (Fernando, et al., 2017). Figure 5, represents the comparison of spectra of commercial fucoidan (*Fucus vesiculosus*) and the extracted crude fucoidan. It was observed that, both the spectra shows much resemblance except few extra peaks signifying the presence of crude fucoidan. The additional peaks may be due to the presence of other monosaccharides and also the structural variation due to the fucoidan from different species.

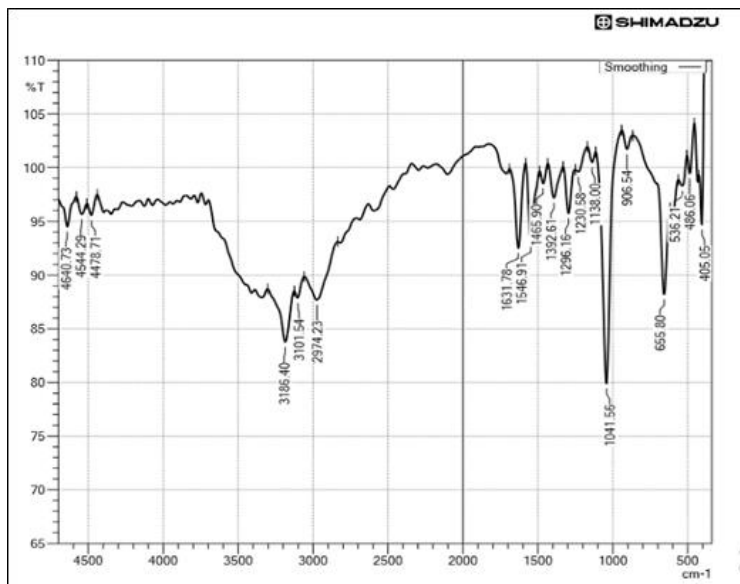


Figure 4. The FTIR spectra obtained for the extracted crude fucoidan shows peaks corresponding to the carboxylic acids, aromatic esters, glycosidic linkages and sulfate groups.

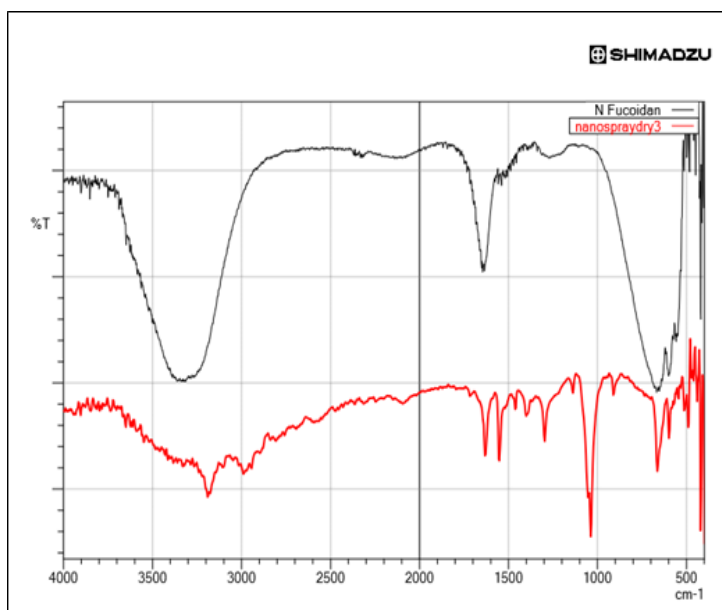


Figure 5. Comparison of FTIR spectra with the commercially available fucoidan shows that, the extracted fucoidan is crude with the presence of other monosaccharides

### 3.5 Antioxidant activity

#### 3.5.1 Free radical scavenging activity

The free radical scavenging activity of the crude fucoidan was determined using DPPH (1, 1-179 diphenyl-2-picrylhydrazyl) radicals and it was compared with the standard ascorbic acid used. From Figure 5 (a), it was observed that, the free radical scavenging activity of crude fucoidan varied between  $19.49 \pm 0.29$  and  $73.33 \pm 0.16\%$  for the concentrations 20-100  $\mu\text{g/ml}$ . Similarly the scavenging activity of the standard ascorbic acid varied between  $24.84 \pm 0.22$  and  $87.99 \pm 0.12\%$  for the same concentration range.



### 3.5.2 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the fucoidan sample was determined using the fenton's reaction and it was then compared with the hydroxyl radical scavenging activity of the standard ascorbic acid. From Figure 6 (b), it was observed that, the hydroxyl radical scavenging activity varied between  $16.99 \pm 2.075$  and  $48.53 \pm 1.55$  % for the varying concentrations 200-1000  $\mu\text{g/ml}$  of crude fucoidan. Similarly the free radical scavenging activity of the standard ascorbic acid varied between  $31.99 \pm 1.576$  and  $73.04 \pm 1.902$  % for the same concentration range.

### 3.5.3 Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of the crude fucoidan sample was determined using the auto-oxidation of pyrogallol and it was then compared with the superoxide anion radical scavenging activity of the standard ascorbic acid. From Figure 6 (c), it was observed that, the free radical scavenging activity varied between  $20.46 \pm 1.539$  and  $64.37 \pm 0.178$  % for the varying concentrations 200-1000 $\mu\text{g/ml}$  of crude fucoidan. Similarly the free radical scavenging activity of the standard ascorbic acid varied between  $31.697 \pm 0.818$  and  $81.56 \pm 1.262$  % for the same concentration range.

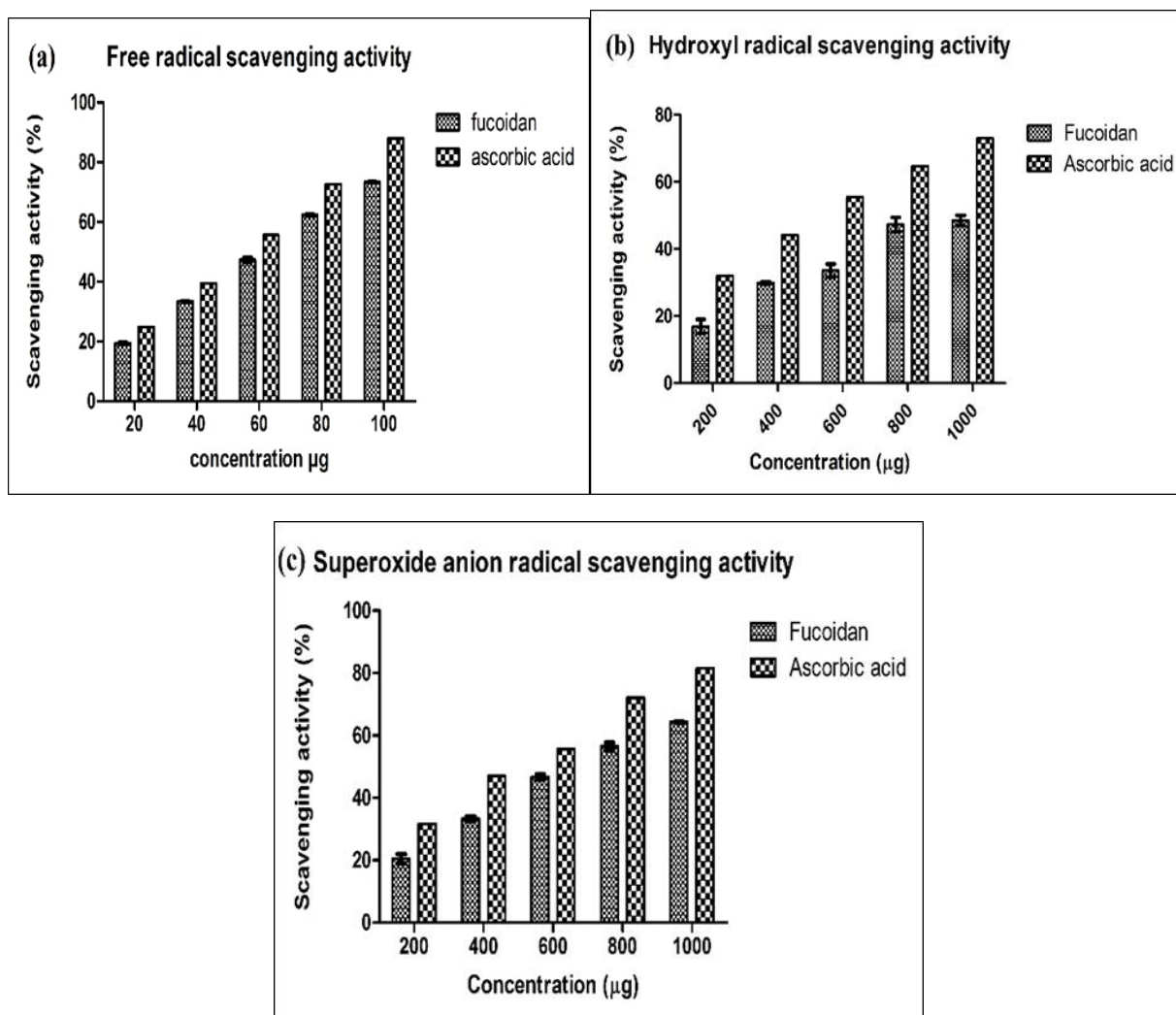


Figure 6. Antioxidant activity of crude fucoidan with standard as ascorbic acid. Crude fucoidan has shown significant levels of antioxidant activity but quite less when compared with the standard ascorbic acid. (a)- Free radical scavenging activity, (b)- Hydroxyl radical scavenging activity, (c)- Superoxide radical scavenging activity.

### 3.5.4 Cell reducing capacity

The cell reducing capacity of the crude fucoidan sample was analysed using MTT assay against cervical cancer cell lines (HeLa cells) and human embryonic kidney cells (HEK). Similarly, the chemotherapeutic drug paclitaxel was used as the standard drug control. From the results and graphs obtained in the Figure 7, it was observed that the crude fucoidan showed significant cell reducing capacity against the HeLa cells with IC<sub>50</sub> concentration of 24 µg/ml (Figure 7 (a)) whereas the IC<sub>50</sub> concentration of paclitaxel against HeLa cells was 30 nM (Figure 7 (b)) Also, the sample and paclitaxel shows cell reducing capacity against HEK cells with the IC<sub>50</sub> concentration of 36 µg/ml (Figure 7 (c)) and 20 nM (Figure 7 (d)). Commercial fucoidan was reported to have cell reducing capacity in HEK cells as high as 84.3 % at 1mg/ml of concentration [22]. Figure 8 shows the control and treated HeLa cells viewed under phase contrast microscope. The cell reducing capacity increases with increase in concentration signifying that the effectiveness is in dose dependent manner.

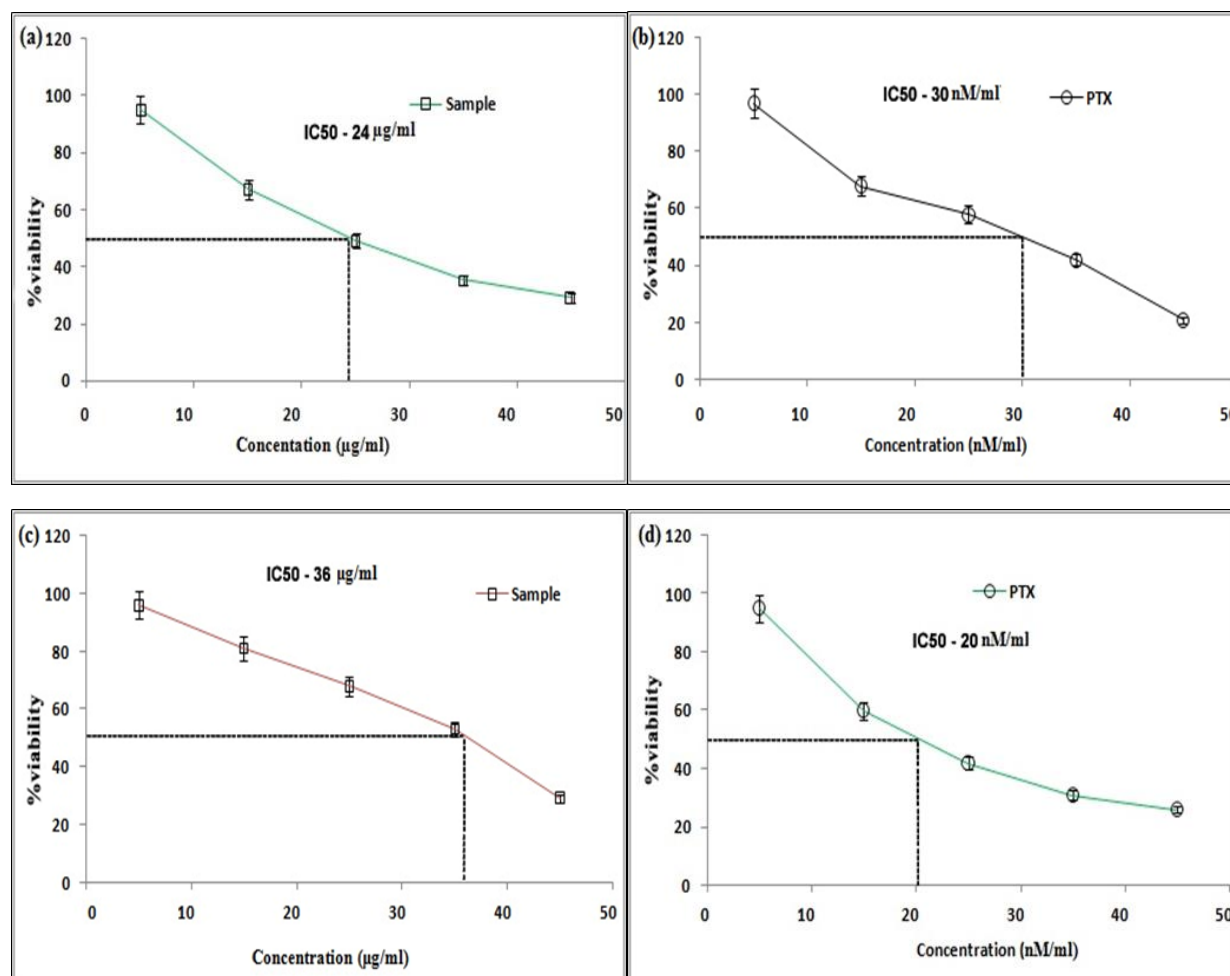
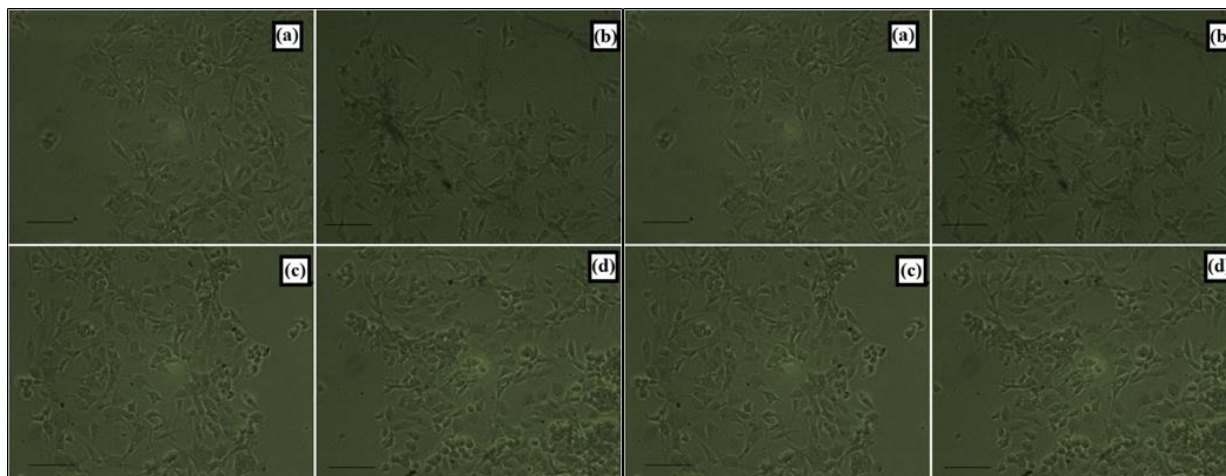


Figure 7. Cell reducing capacity of crude fucoidan and paclitaxel.(a)-crude fucoidan against HeLa cells has IC<sub>50</sub> value of 24µg/ml (b)-paclitaxel against HeLa cells has IC<sub>50</sub> value of 30nM (c)-crude fucoidan against HEK cells has IC<sub>50</sub> value of 36µg/ml (d)-paclitaxel against HEK cells has IC<sub>50</sub> value of 20nM



**Figure 8.** HeLa cells viewed under phase contrast microscope treated by crude fucoidan and paclitaxel.(a)-control, (b)-10 µg/ml, (c)-20 µg/ml & (d)-40 µg/ml

#### 4. DISCUSSION

Fucoidans are sulfated heteropolysaccharides generally found in marine brown algae and some marine invertebrates. It is mainly composed of L-fucose and sulfate ester groups (Isnansetyo, Lutfia, Nursid, Trijoko, & Susidarti, 2017). In addition, it also contains other monosaccharides such as galactose, glucose, mannose, rhamnose, arabinose and xylose, uronic acids, proteins and acetyl groups (Li, Lu, Wei, & Zhao, 2008) & (Wang, et al., 2019). The chemical composition of fucoidans varies depending on the species, extraction methods and season collection which in turn influences its activities (Cumashi, et al., 2007), (Wang & Chen, 2016) & (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010). The composition of monosaccharide and sulfate, molecular weight and structure of main polymer chain of fucoidan influences the biological activities of sulfated polysaccharides to a great extent. Although in this study, the crude fucoidan is characterized and compared with commercial fucoidan using FTIR, the structural variations cannot be understood in detail. In regard to this, the structural variations in the extracted fucoidan can be determined using a technique that provides detailed information about the fucoidan's structure like NMR for further reference. Similarly determination of sulfate content and molecular weight of the crude fucoidan can be correlated with its antioxidant activity and cell reducing capacity.

Also, it was reported that, fucoidan with high sulfate content and low molecular weight possessed high degree of antitumor and other biological activities. Whereas this is vice versa in few rare cases of fucoidan depending on the species used for extraction (Anastyuk, et al., 2012). Hence further studies can be done by concentrating and analyzing low molecular weight fucoidans for determining any changes in the antioxidant and antitumor activity is observed.

Only a small amount of fucoidans administered used to cross the intestinal walls whereas almost all fucoidan molecules tends to accumulate in kidneys which is then later excreted in urine and this accumulation might be reason for the toxicity of fucoidan in kidney cells (Chen, et al., 2017) & (Udani, 2012). Both extracted and commercial fucoidan had shown toxicity against kidney cells (HEK) in a study performed by Mak, et al., 2014. This explains the cell reducing capacity of the extracted crude fucoidan from *Sargassum* species in the HEK cells. In addition, paclitaxel, the chemotherapeutic drug control used had also shown toxicity against HEK cells. However, fucoidan shown more toxicity against cancer cells than kidney cells with cancer cells having less IC<sub>50</sub> value. The accumulation in kidneys can be overcome by increasing the bioavailability of fucoidan by combining it with other compounds with high bioavailability.

#### 5. CONCLUSION

The polysaccharide fraction was extracted from *Sargassum* species and fractionated using anion exchange chromatography yielded crude fucoidan. The crude fucoidan was characterized using FTIR and the spectra obtained shows the characteristic peaks of fucoidan along with additional peaks signifying that further purification using gel permeation chromatography is needed to eliminate other polysaccharides and monomers. The crude fucoidan showed

significant results in their free radical scavenging activity determined through DPPH with scavenging percentage of  $73.33 \pm 0.16$  % at 100  $\mu\text{g/ml}$ , hydroxyl radical with scavenging percentage of  $48.53 \pm 1.55$  % at 1000  $\mu\text{g/ml}$  and superoxide anion radical scavenging assay with scavenging percentage of  $64.37 \pm 0.178$  % at 1000  $\mu\text{g/ml}$ . Further, in MTT assay the crude fucoidan showed cell reducing capacity in HeLa cells with IC<sub>50</sub> value of 24  $\mu\text{g/ml}$  and IC<sub>50</sub> value of 36  $\mu\text{g/ml}$  in HEK cells. The cell reducing capacity of the crude fucoidan against HEK cells was assumed to be due to the low molecular weight fucoidan fraction and other monomers present in it and needs further investigation with purified fucoidan. Since, the high molecular weight fucoidan is assumed to be spherical unlike low molecular weight fucoidan which is linear, the sulfate groups doesn't have much exposure to cancer cells and the cell reducing capacity against cancer cells was reported to be linked with high sulfate content (Mak, et al., 2014). Hence the cell reducing capacity of the crude fucoidan against cancer cells can be increased by purification and combining it with gallic acid for enhanced bioavailability which prevents the accumulation in kidneys and promotes significant level of cell reducing capacity against cancer cells.

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