Characterisation of exopolysaccharides isolated from *Dunaliella salina* exhibiting immunomodulation in peripheral blood mononuclear cells and RAW 264.7 macrophages

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By

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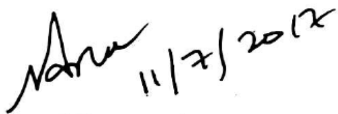
Certificate

This is to certify that the project entitled "**Characterisation of exopolysaccharides isolated from *Dunaliella salina* exhibiting immunomodulation in peripheral blood mononuclear cells and RAW 264.7 macrophages**" submitted by Mehendi Goyal in the partial fulfilment of the requirement for the award of degree of Master of Technology in Biotechnology to Department of Biotechnology, Thapar University, Patiala, is a record of student's own work carried by her. The report has not been submitted for the award of any degree or certificate in this or any other University or Institute.



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Candidate Declaration

I, hereby declare that the work presented in the thesis entitled "**Characterisation of exopolysaccharides isolated from *Dunaliella salina* exhibiting immunomodulation in peripheral blood mononuclear cells and RAW 264.7 macrophages**" in the partial fulfilment of the requirement for award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my work during the period of one year from June 2016 to June 2017, under the guidance of Dr. Manoj Baranwal, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other diploma or degree.

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Abstract

Dunaliella salina (D. salina) is the green halotolerant algae known for its growth in hypersaline environment. Recently, the algae have been explored for the extracellular polysaccharides (EPS) and their various industrial applications. The scope of this study is characterization and immunological assessment on peripheral blood mononuclear cells and RAW 264.7 of EPS isolated from *D. Salina* and enhancement of the production of EPS by cultivating in stress conditions. Column chromatography of crude EPS resulted in five fractions where DsF1 and DsF4 were observed to contain high sugar content. Cell proliferation assay showed that DsF1 and DsF4 increased the cell growth of PBMC in a dose dependent manner. Three cytokines (IFN-γ, TNF-α, TGF-β) were also estimated in PBMC culture by ELISA and observed to be increased with increase in concentration for DsF1 and DsF4 indicating the immunostimulatory effect. In case of RAW cells, both DsF1 and DsF4 showed a decreasing trend of cell growth and nitric oxide production (griess reagent) with increasing concentration. Mass spectroscopy results revealed the presence of hetero polysaccharides composed of various hexoses (glucose, galactose, and fructose) and pentoses (xylose) both in DsF1 and DsF4. The presence of halide associated polysaccharides was also suggested which was confirmed by FTIR analysis. Oligosaccharides (eight monosaccharide units) were also found to be present in fraction DsF4 as per the mass spectroscopy results. FTIR analysis have shown the presence of proteoglycans due to the detection of N=O and N-H group. Enhanced production of EPS was obtained when grown in stress conditions i.e. nitrate (KNO_3:14.7 mM and 24.5 mM), phosphate (KH_2PO_4: 4.2 mM and 7 mM) and sodium salt (NaCl: 3 M and 3.5 M) stress as compared with normal conditions (KNO_3: 4.9 mM, KH_2PO_4: 1.4 mM and NaCl: 1.7 M).Hence, it is concluded that two fractionated EPS isolated from *D. salina* possess immunomodulatory activities and has the potential to be used for therapeutic purpose.

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Chapter I

Introduction

1 Introduction

Ever since the era of biotechnology started, scientists have been searching out ways to utilise living cells for the use of mankind. Emerging economies and growing world population is suffering from a lot of major issues such as energy sources, food insecurity and health conditions. For the last decade, use of micro algae has drawn attention in search of biotechnological and biopharmaceutical products. A variety of microalgae such as *Chlorella*, *Dunaliella, Nostoc, Spirullina* and *Ulva* have already been reported to be cultivated on the commercial level in order to achieve the desired outcome (Wolkers *et al.* 2011).

Dunaliella salina; unicellular, biflagellate, green, halotolerant microalga is known to survive in the wide range of salts (Borowitzka & Siva 2007). It belongs to the phylum Chlorophyta, order Volvocales and family Polyblepharidaceae mostly distinguished by a rigid cell membrane (osmoregulation), absence of cell wall, presence of plastids (photosynthesis) and two flagellas (motility) (Borowitzka & Siva 2007). The alga is known to possess various bioactive properties such as anti-viral, anti-bacterial, anti-cancer and immunomodulatory activities (Hosseini Tafreshi & Shariati 2009). These bioactive compounds are mostly produced intracellularly which have gained commercial importance such as trans and cis-βcarotene, fatty acids (oleic acid, linolenic acid and palmitic acid) and glycerol. Recently, the extracellular polymers of microalgae have received attention by the scientific community. The extracellular secretion is composed mainly of hetero-polysaccharides which are reported to play an advantageous role in various industries such as cosmetics, food, biomedical and pharmaceuticals (de Jesus Raposo *et al.* 2015b). Several species of microalgae such as *Chlorella, Spirullina, Nostoc* have been known to produce polysaccharides extracellularly (de Jesus Raposo *et al.* 2015b).

Immuno-modulation refers to any change involving induction, amplification, expression or inhibition of any particular part or the phase of the immune response. Immunomodulators may be categorised as immunosuppressants and immunostimulants based on their effects on the modulation of immune system. Polysaccharides have been reported to act as potential immunomodulators and various microalgae are explored for these biopolymers (de Jesus Raposo *et al.* 2015a). Soluble polysaccharides (sPS) isolated from *Chlorella stigmatophora* have showed immunosuppressive effect on macrophage cells whereas the immunostimulant effect was observed for the exopolysaccharides isolated from *Phaeodactylum tricornutum*(Guzman *et al*. 2003). The nitric oxide production from the macrophages have been tested and it had been found that soluble polysaccharides (sPS) from *Gyrodinium*

impudicum termed as pKG 03 activated the production of nitric oxide and stimulated the production of cytokines from mouse macrophage cell lines *(*Bae *et al.* 2006*)*. Although, many microalgae have confirmed for their bioactive extracellular polysaccharide secretions, still, no algal polysaccharide is commercially exploited.

Recently, due to the diverse applications possessed by microalgae, more research is going on to explore the products obtained from microalgae. *D. salina* is a commercially important organism which is mostly explored for intracellular products. Like other algae from the Chlorophyta division, *D. salina* has also explored previously for the secretion of extracellular polysaccharides and various industrial applications have been reported (Hosseini Tafreshi & Shariati 2009). However, extracellular polysaccharides (EPS)'s bioactivity is mostly reported for other microalgae. In the present study, the EPS isolated from *D. salina* were assessed for the growth on peripheral blood mononuclear cell (PBMC) and RAW 264.7 mouse macrophage cell lines. Further, EPS were analyzed by thin layer chromatography (TLC) and fractionated by silica gel chromatography. Fractions having highest sugar content were assessed for their cell growth effect on PBMC and RAW 264.7 cells, nitric oxide production from RAW cells and cytokine release from PBMC. The fractions were characterised by mass spectroscopy (MS) and Fourier transform infrared spectroscopy (FTIR) to identify the masses and functional groups of the compounds present in the fractions. Then, the culture was grown in three different stress conditions in order to enhance the production of the polysaccharide content.

As polysaccharides also are commercially important products with various applications, isolation of polysaccharides from an organism whose market is already established would be highly economical.

Chapter II

Review of Literature

2. Review of Literature

2.1. Algae backdrop

Algae predominantly come under the group of aquatic photosynthetic organisms. This vast autotrophic group includes the organisms as small as micromonas species which is not even visible to the naked eye and the organisms as giant as kelps that reach 60 meter in height. The organisms are known to produce complex compounds from simpler inorganic chemicals and light. Algae plays very important role in maintaining the ecology by producing oxygen. It also acts as a very important member of the aquatic as well as land food chain. Several algae have gained the economical importance as the source of oil, pharmaceuticals and other industrial products (Madigan *et al.* 1997).

Early in 1830, algae were classified under protists as red, brown and green on the basis of the reflection of different chloroplast pigments such as carotenoids, phycobillins and chlorophyll. Later, phylogenetic analysis of algae opened new horizons of the classification as photosynthetic green algae are classified under kingdom plantae but blue green algae (cynobacteria) are classified as prokaryotes. Algae are divided into several types based on the complexity of morphology as parenchymatous, coenocytic, planktonic, neustonic, cryophilic, thermophillic, edaphic, epizoic, epiphytic, corticolous, epilithic and chasmolithic algae (Madigan *et al.* 1997).

Planktonic algae or phytoplanktons are defined as the algae which resides near or inside water (fresh or salt water) and shares the feature of photosynthesis with complex plants. This type of algae consists of chlorophyll and other accessory pigments such as phycobilins or carotenoids in their cells which allows them to convert small inorganic molecules into complex organic compounds. Phytoplanktons can be classified as macroscopic or microscopic algae depending upon the size. The planktonic algae is further classified into several classes such as blue green algae (Cyanophyta), green algae (Chlorophyta), yellow green algae (Xanthophyceae), golden brown algae (Chrysophycea), brown algae (Phaeophyta), red algae (Rhodophyta), diatoms (Bacillariophycea), cryptomonads (Cryptomonadineae), dinoflagellates (Dinophyceae) and euglenoids (Euglenophyceae) (Madigan *et al.* 1997).

Chlorophyta is comprised of around 15000 species. The morphology of Chlorophytes is characterised by the green motile or non-motile algae and the presence of a double cell wall made of cellulose and pectin. The chlorophyll pigments such as chlorophyll a and b, carotene

and xanthophyll are present in the cell organelle plastid. The division is further classified in various classes depending upon size and shape such as unicellular (*Chlamydomonas*), filamentous (*Spirogyra*), colonial (*Volvox*) and tubular (*Actebularia*) (Madigan *et al.* 1997).

2.2. Ecological and commercial importance of algae

Algae have been considered ecological important species provided the fact that it is involved in photosynthesis and releasing oxygen. Since ancient times, humans are aware of the algal importance and have included the species as an integral part of their daily routine. These days, researchers are more inclined towards finding other potential uses of microorganisms as they are easy to manipulate and grow. Different varieties of algae have been commercialised as food such as *Porphyra, Spirogyra* and *Laminarta* which are the integral part of Japanese food. Other species of algae such as *Chlorella, Dulse and Undaria* are also consumed in Canada, Korea and China extensively as the integral part of their diet. Many algal species have been taken as multivitamin and mineral supplements which are consumed extensively in the form of chewing tablets (Chu 2012).

Industrially, diatoms are used in soap and sugar manufacturing. It has also gained importance in cement industry due to the strong and stable structures formed by algal communities (Priyadarshani & Rath 2012). Apart from these industries, algae have also been used in textile industry (agar agar from *Gelidium*), agar isolated from *Chondrius* and *Careragenium* is widely used as the stiffening agent in cosmetics industry. *Chara* and *Nitella* have been known to have inhibitory effect against malarial parasite. Antibiotic chlorellin obtained from green alga *Chlorella* is very effective against gram negative bacteria (Amaro *et al.* 2011).

Various products such as tablets, capsules, powders obtained from various algae have been commercialised worldwide bagging the consumption of more than 1000 tonnes per year. One of the examples is Earthrise nutritionals, California, USA which are producing beverages and other food items from *Spirogyra* (3000 tonnes/year). Likewise, Australian company, Cognis Nutrition and health are producing β-carotene from *Dunaliella salina (D. salina)* (1200 tonnes per year) (Priyadarshani & Rath 2012).

Current study takes into account a photosynthetic eukaryotic alga, *D. salina* which is the most halototerant eukaryotic algae as it is known to survive even in the saturated salt concentrations. *D. salina* is commercialised for glycerol, a major metabolic product which is required for its stabilisation in hyper-salt environment and β-carotene.

2.3. *Taxonomic elucidation of Dunaliella salina*

Dunaliella salina (D. salina), belongs to phylum Chlorophyta, order Volvocales, family Polyblepharidaceae. It is the unicellular, biflagellate green algae characterised by the absence of cell wall, single cup shaped chloroplast and a central pyrenoid. It was first sighted in 1838 on the coast of France by Micheal Felix Dunal and rediscovered in 1905 by Tredesco. Firstly, it was described as a unicellular, red coloured halotolerant algae primarily termed as *Hematococcus salina*. *Dunaliella* genus is composed of 29 species such as *Dunaliella tertiolecta, Dunaliella primolecta, Dunaliella virdis, Dunlaiella bioculata* and *Dunaliella media* based on the different morphological and division patterns. The algae holds economical importance as it is involved in producing β-carotene and glycerol intracellularly (Borowitzka & Siva 2007).

2.4. *Nutrition of D. salina*

For the growth of *D. salina,* different parameters are required. Although the organism is adapted to grow in different stress conditions, but still, needs the different factors as a supplement in the media to sustain growth which are mentioned below

Light: Light is the primary requirement of the organism as it is photosynthetic algae and requires as an energy source for its metabolism. The source of light vary depending upon the type of culture for instance if the culture is open, the natural sunlight act as the source of light but if the algae is grown in bioreactors or in other closed systems, then different light sources such as white fluorescent lamps are required (Hosseini Tafreshi & Shariati 2009). It is reported that the growth of algae and the production of metabolites is dependent on the intensity and duration of light provided (Ben-Amotz & Avron 1989). It was observed that carotenoid production increased when high frequency light was used as the source.

Temperature: Temperature plays a very pivotal role in the growth of the organism although the algae has adapted to survive in the wide range of temperature i.e. 0-45ºC (Hosseini Tafreshi & Shariati 2009). The optimum temperature for the growth of *D. salina* is reported to be 25-32ºC (Ben-Amotz 1995). In closed systems, the temperature is controlled by a thermostat whereas it is impossible to control in open ponds that is why low night temperatures have caused the cells to grow at slow rate. It is observed that at higher temperatures (>45ºC), glycerol leaks out from the cells and it gives bacteria and fungus to grow in the culture. However, at 40ºC, the carotenoid production is increased (Borowitzka & Borowitzka 1988).

pH control*: *D. salina is adapted to sustain its life in the pH range of 0-11 however, it is always recommended to keep the pH less than 8 and optimum pH is maintained to 7.5 ± 0.2

in the cultures as high pH stunt the growth of algae. The pH is maintained in open ponds by the addition of HCl as the pH increases due to the photosynthetic fixation of carbon dioxide which leads to the release of more hydroxide ions (Ben-Amotz & Avron 1989).

Nutrient requirements: The algae like other organisms require the carbon and nitrogen sources to grow. As *D. salina* is a photosynthetic alga, it requires only CO_2 and bicarbonate as the sole carbon sources. CO_2 bubbles are passed in the culture at the rate of 0.41/mL whereas bicarbonate is supplemented as $NaHCO_3$ at the concentration of 10 mM/L (García-González *et al.* 2003). Nitrate act as the best nitrogen source for the growth of the organism. Reduction in the nitrogen source may induce stress over the cells and cause the cells to produce more metabolites such as carotenoids but nitrate limitation beyond threshold will lead to shunted growth and may even lead to cell death. Cell death is the primary reason why other nitrogen sources such as urea and ammonium salts are not used (Hosseini Tafreshi & Shariati 2009). KH_2PO_4 in the optimum concentration of 0.2 µg/L is the best source for the supplement of potassium (Gibor 1956). Likewise, high quantity of sulphate (2 mM/L) is also required for the growth of algal cells (Ben-Amotz & Avron 1989). Other macro-elements which are good for the growth of the cells include potassium, calcium, magnesium, chloride and sodium ions. Certain microelements such as zinc, manganese, cobalt and copper are also required as nutrition for the growth of the algal cells (Borowitzka 1990).

2.5. Reproduction in D. salina

Both vegetative and sexual reproduction is observed in *Dunaliella*. At high salinity, the organism adapts to vegetative reproduction. The commencement of cell division is followed by the furrowing of cell membrane starting with the anterior flagellar end and reaching to the posterior end. Followed by the furrowing, the chloroplast and pyrenoids divide while the cells are connected through a cytoplasmic bridge. The daughter cells start to form their own flagella as cytoplasmic bridge breaks i.e. before the cells actually divide. At low salinity (>10%), green cells are accumulated and palmella stage is observed where they lose the flagella and eyespot. When these palmella cells are grown in normal salinity, they regrow their flagella and regain its motile nature. The cells of this organism are also known for the formation of cyst like structures known as aplanospores characterised by the two layered thick fucose wall. These vegetable cysts are observed in various stress conditions such as salinity, nitrogen, temperature, light and sulphate stress (Oren 2014). Different species of *Dunaliella* genus such as *D. teritolata, D. virdis, D. salina and D. terricola* have been observed for the formation of aplanospores (Oren 2005).

Sexual reproduction is induced by low salinity. The mating is done when two different forms of same specie (+/-) joins their flagellae and forms the mating tube. The anterio-lateral fusion of two cells leads to the formation of tetra-flagellate structure known as planozygote. It is followed by zygote germination where 4, 8, 16 or 32 cells are formed as the result of meiotic division. The major factor which is required for the sexual reproduction is size of the cell. The small cells formed a structure called palmellae which are transformed into gametes as they grow. After around 20-30 min, the gametes fuse and form zygote which undergoes meiotic divisions to form 4-8 cells. On the other hand, the larger cells do not need to enter the palmellae stage and form the tetra flagellate structure. It was observed that the cells must attain a particular size to be sexually competent (Oren 2014).

2.6. Mechanism of sustaining salt stress

Species of genus *Dunaliella* are known to survive in a wide range of salts and this is achieved by maintaining the osmotic ionic balance. The cell requires certain mechanisms for enabling their survival in such halophilic conditions. The cells are known to change their volume as well as shape in response to hyper/hypo osmotic conditions (Chen & Jiang 2009). Glycerol is known to accumulate in the organism when cells are salt stressed and the metabolite has been commercialised as glycerine in food and pharmaceutical industry. The osmoregulation process in *Dunaliella salina* is divided into three phases

1) Responding with either decrease or increase in shape and size of cells to prevent the cells from apoptosis which occurs in first 10-15 min of stress.

2) Second stage is the process which takes place in another 3-4h where intracellular osmotic pressure is maintained by intracellular regulation of glycerol concentration.

3) Last stage refers to the long term stress condition where the cell activates genes which lead to the accumulation of salt induced proteins. This happens after the cell has been in stress condition for 12-24h.

2.7. Osmotic arrangement

Ions required for the osmotic arrangement: All cells maintain an intracellular level of 20-100 mM Na^+ concentration which is mostly less than outside environment (1-5 M) showing that the cells are effective enough to exclude excess ions to enter the cell (Chen & Jiang 2009). The extrusion is mediated by the Na^+/H^+ antiporters by ATP mediated fashion. Likewise, K^+ ions concentrations are maintained as 100-200 mM via the K^+ transporters (Figure 2.1).

Osmoregulation is also regulated by maintaining the glycerol levels in the algal cells intracellularly. The glycerol is formed by two mechanisms i.e. by using photosynthetic

products or by starch degradation (Ben-Amotz 1980). Various enzymatic reactions follow to accumulate glycerol in the cells. Glycerol is an effective osmoregulator as it is the highly soluble and an end product metabolite (Figure 2.2).

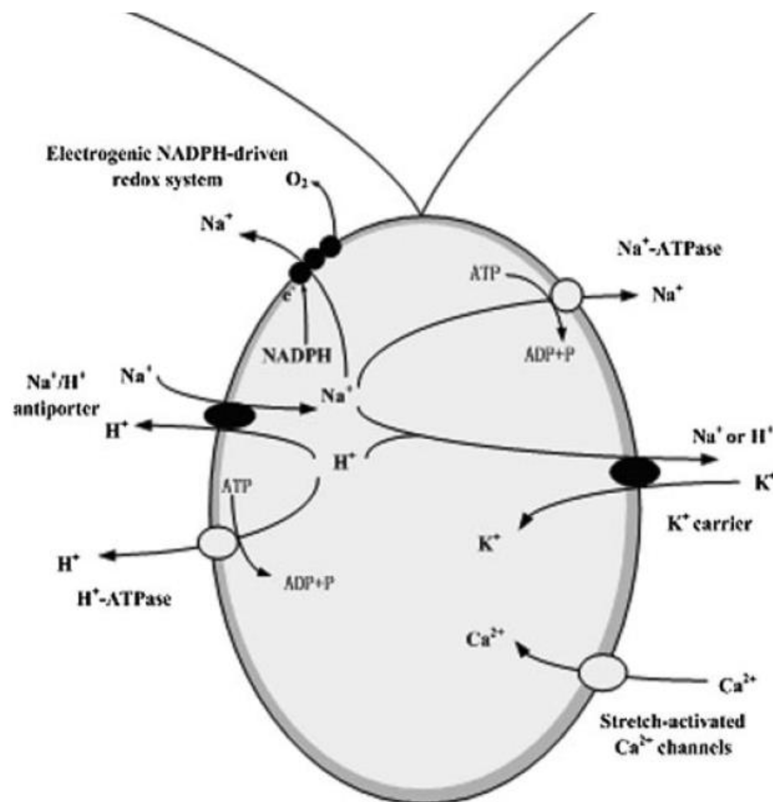

Figure 2.1: Mechanism of osmoregulation by controlling the ion exchange through ion channels (Chen & Jiang 2009)

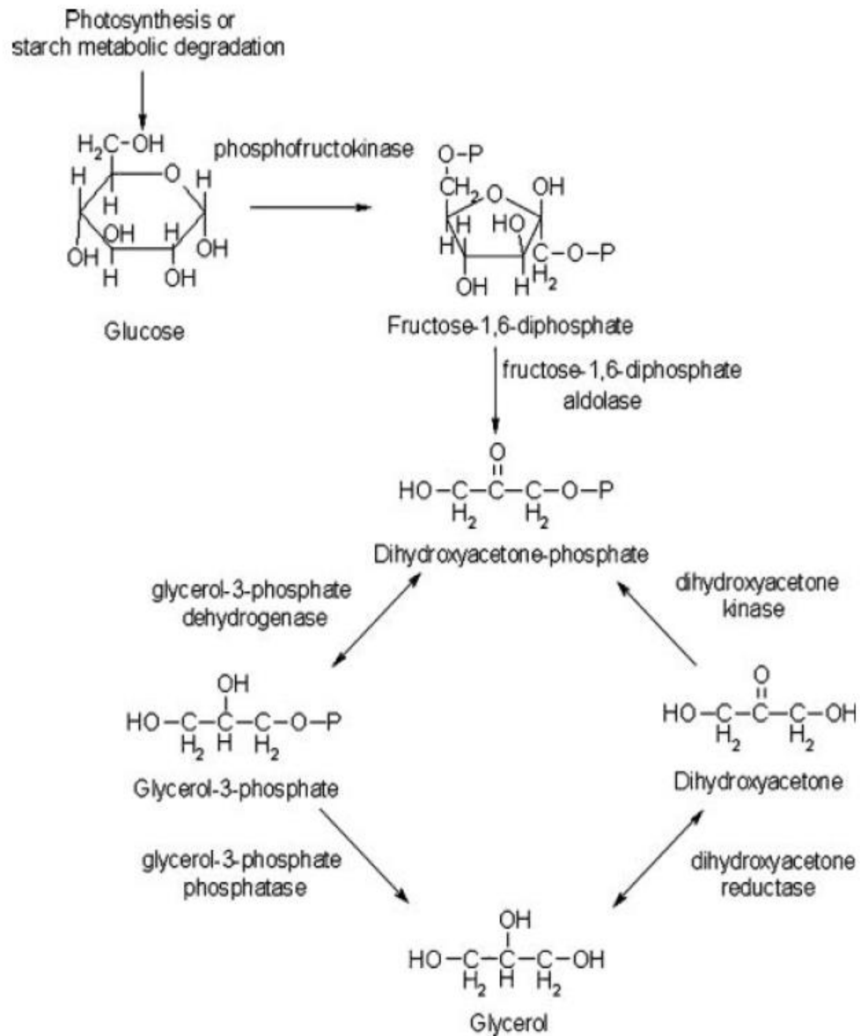

Figure 2.2: Mechanism of production of glycerol (Ben-Amotz 1980)

2.8. Commercialisation of D. salina

Commercial production of *D. salina* is carried out extensively either in unstirred open ponds or intensively in paddle wheel ponds. Photobioreactors plc, UK has also proposed for establishment of plant in Spain which carries out the production in large tubular bioreactors (García-González *et al.* 2003). Right now, there are two producers of the algae on a commercial basis; Western Biotechnology ltd, Perth, Australia (5 hectare area) and Betatene ltd, Melbourne, Victoria, Australia (50 hectare area) (Curtain 2000). Alternatively, the biomass of the culture is increased by using paddlewheel ponds commercially used by companies in Israel and UK (Hosseini Tafreshi & Shariati 2009). Closed cultures pose several advantages of preventing the contamination with other protozoans but as they are expensive than open lagoons, it is not preferred although, better designs are awaited. The specific increase or decrease in nutrient stresses leads to either increase or decrease in the

production of a specific metabolite thus, it becomes important to know the growth requirements of the algal cells. When any product is commercialised, cost of production plays a very important role. In any bioprocess, downstream processing (extraction and purification) mostly adds up to the cost of the final product. β-carotene and glycerol being intracellular products pose a lot of difficulty in extraction and purification as different down streaming processes leads to the destabilisation of the product and a high shear stress caused leads to the non-reusability of the culture. Despite of these problems, β-carotene demand is increased and is sold for $300 per kg (Ben-Amotz & Avron 1989).

Alternatively, in spite of extracting compounds, *D. salina* is also marketed as a whole organism under a trade name of Algotene® by Inter Clinical laboratories Pty Ltd. Australia (UltraHealth *et al.*). Previously, *Spirullina* and *Chlorella* have been considered fit for the consumption as whole organism and have been commercialised as capsules (Ciferri 1983). Algotene is the dried *D. salina* organism packed as capsules. The product is a rich source of various nutrients such as proteins, carbohydrates, fats, minerals, β and α carotene, lutein, zeaxanthin and cryptoxanthin. *Dunaliella* is the better nutritional source as compared to *Spirullina* (Table 2.1).

Apart from intracellular carotenoids and glycerol, algae are also explored for their extracellular secretions and it has been found that majorly, the secretions comprise of polysaccharides. Recently, various industrial applications have been associated with extracellular polysaccharides (de Jesus Raposo *et al.* 2015a). One of the great advantages of these secretions is that being extracellular; the cost of extraction and purification is decreased in contrast with the intracellular productions. Also, the culture is reproducible as no damage to the cells occurs in the downstream processing.

Table 2.1: Nutritional analysis of commercialised *D. salina* (Algotene) and *Spirullina*

Nutrition	*D. salina*(per 100 g)	*Spirullina*(per 100 g)
Proteins	7.4 g	57 g
Carbohydrates	29.7 g	24 g
Fats (total)	7 g	8 g
Minerals	49 g	7 g
Energy	893 kj	121.4 kj
β-carotenoids	1100-2100 mg	0.342 mg
α-carotenoids	53.1-102.4 mg	0.035 mg
Lutein, Zeaxanthin	54.3 - 46.5 mg	0.002 mg
Cryptoxanthin	23.4- 46.5 mg	0

Source: (http://www.australiasown.com.au/algotenebrochure.pdf

2.9. Bioactivity of D. salina

β-carotene: It is required in proper functioning of vision which is accomplished by its oxidation into vitamin A. The product is commercially isolated from *D. salina* as this particular carotenoid holds applications in food, biopharmaceuticals and cosmetics industry as colorants and anti-oxidants. Further, β-carotene has also been found to inhibit the growth of tumours and controlling cardiovascular problems. Although β-carotene is also obtained from the vegetables but algae can store 1000x more β-carotene intracellularly (Ben-Amotz & Avron 1989). Commercially, various concentrations of β-carotene liquid are available (4% as dietary supplement, 2% in beverages and 2-5 % in aquaculture feed) (Hasan & Rina 2009).

Other carotenoids such as phytoene, phytofluene, lutein and zeaxanthin have cancer preventing and anti-oxidant activities. Lutein is also reported to inhibit certain chronic diseases such as cataract (Pulz & Gross 2004).

Dried Algal meal: After the removal of β-carotene and glycerol from the cells, the dried algae are consisted of about 40% protein. The amino acid composition of the proteins is low in cysteine, isoleucine, methionine and tryptophan but relatively high in lysine. *D. tertiolecta* has been extensively used as a source of single cell protein. Along with proteins, a high concentration of total lipids rich in ώ- polyunsaturated fatty acids are also reported in the

dried algal mass which is a very good source of anti-oxidants (Hosseini Tafreshi & Shariati 2009).

Apart from the bioactive compounds which are either anti-cancer or possess good anti-oxidant activity, other valuable compounds such as the enzymes and vitamins are also isolated from *D. salina*. Dihydroxyacetone reductase enzyme is commercially sold which have been isolated from *D. salina* (Ben-Amotz 1980).

Other species of genus *Dunaliella* such as *D. tertiolecta* has shown various bioactive properties such as analgesic, antihypertensive, antioedema, bronchodilator, polysynoptic block and muscle relaxant activities (de Morais *et al.* 2015). *D. primolecta* has effectively shown inhibition against *Taphylococcus aureus*, *Bacillus cereus*, *Bacillus substilis* and *Enterobacter acrogens*. A study concluded that the 15 different volatile compounds and the fatty acids are responsible for the anti- microbial effect observed in case of *D. salina* (Herrero *et al.* 2006). The study involved the assessment of organic extracts of *D. salina* (petroleum ether, hexane and ethanol) against the growth of *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* to find that the extracts were effectively inhibiting their growth.

2.10. Polysaccharides

Polysaccharides are the carbohydrate polymers which are composed of mono-saccharide units. The mono-units polymerises as one water molecule escapes creating α-1,4 glycosidic linkages (Nelson *et al.* 2008). Polysaccharides are also characterised by branching as the molecule involves more mono saccharide units. Branching is achieved by α-1,6 glycosidic linkages. Polysaccharides are classified as homopolymers (polymers of single monosaccharide units) such as glycogen and starch (polymers of glucose) and heteropolymers (polymers of different monosaccharide units) such as hyaluronic acid (polymer of D-glucuronic acid and N-acetylglucosamine) (Figure 2.3, 2.4). Polysaccharides in natural existence are involved in variety of functions such as energy storage (glycogen, starch) and structural (cellulose, chitin).

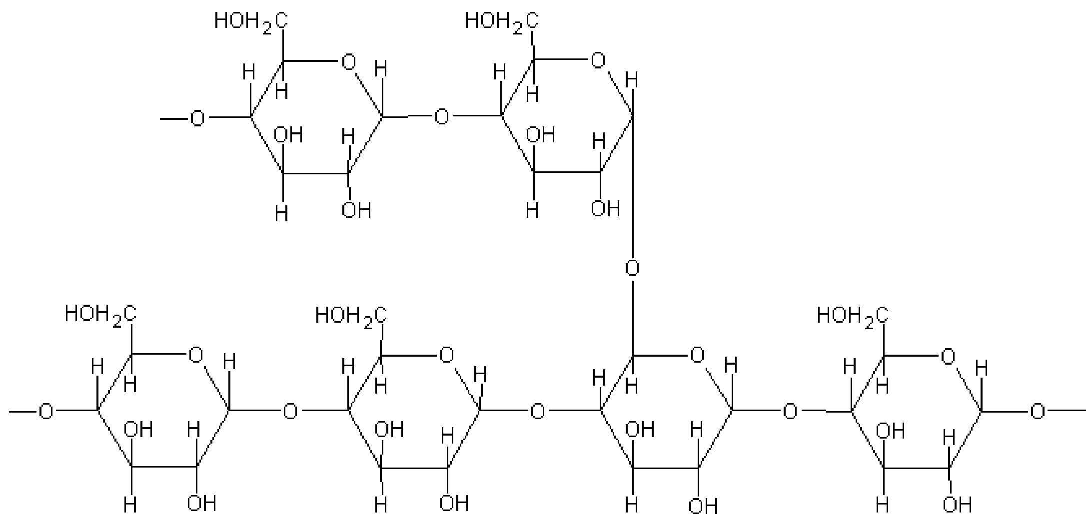

Figure 2.3: Glycogen homo-polymer with repeating glucose units linked by α-1, 4glycosidic bonds and branched by β-1, 6 linkage

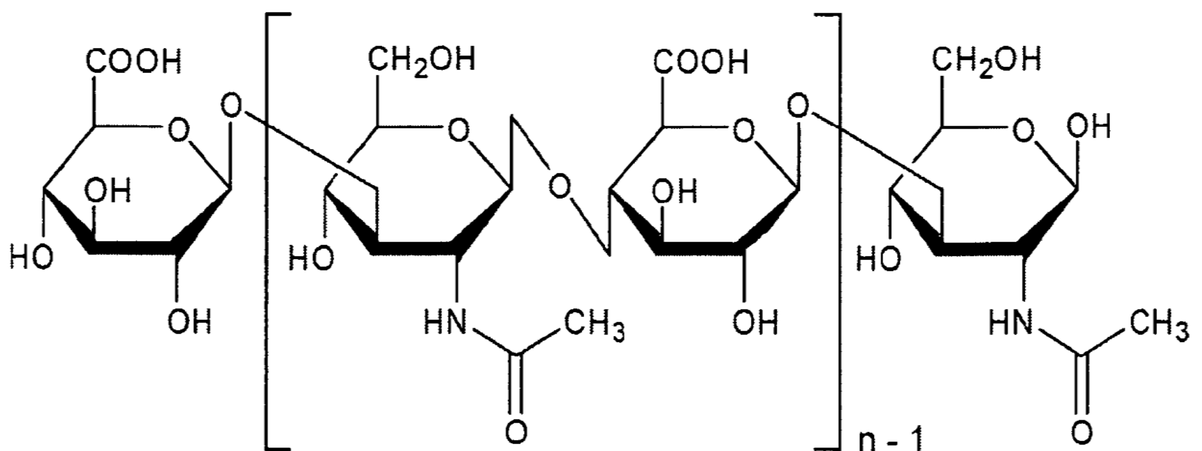

Figure 2.4: Hyaluronic acid hetero-polymer composed of D-glucuronic acid and N-acetylglucosamine

2.11. Polysaccharides isolated from various algae

Microorganisms are exploited for the extracellular production of polysaccharides. Algae is known to contain high amount of polysaccharides mainly classified based on the function performed (structural, mucopolysaccharide, and storage) (Madigan *et al.* 1997). Species such as *Ascophyllum*, *Porphyra* and *Palmaria* have been found to contain very high amount of sugar content (76% of the total dry weight) (Kraan 2012). Presence of polysaccharide differs as the species changes for example, green algae are reported to contain sulphated galatans, xylans and halide associated polysaccharides whereas brown algae contain alginic acid,

fucoidan, laminarin and sargassan. Similarly, red algae are possessed with carrageenan, agars, xylans, flordean, starch and galactan (de Jesus Raposo *et al.* 2015a). Mostly, the polysaccharides (PS) isolated for the algae are heteropolysaccharides except the soluble polysaccharides (sPS) isolated from *Gyrodinium impudicum* (homopolymer of galactose) and PS from *Chlorella vulgaris*, β-1,3 glucan (homopolymer of D-glucose) (Yim *et al.* 2007). The heteropolymers isolated from other algae were reported to be mainly composed of xylose, glucose and galactose in different proportions. However, other mono saccharides such as rhamnose, fucose or mannose may also be found (Table2.2).

Table 2.2: Extracellular polysaccharides secreted by various micro algae (de Jesus Raposo *et al.* 2015a)

Specie	Group of Algae	Type of polysaccharide	Monosaccharide units
Phaeodactylum tricornutum	Diatoms	sEPS	Glucose, xylose, mannose, rhamnose
Chlorella stigmatophora	Chlorophytes	sPS	Glucose, xylose fucose
Chlorella. Vulgaris	Chlorophytes	PS (β-1,3 glucan)	Rhamnose, arabinose, galactose, 2-O methyl Rhamnose
Dunaliella salina	Chlorophytes	EPS	Galactose, glucose, xylose, fructose
Porphyridium	Rhodophytes	sPS	Xylose, galactose, glucose
Gyrodinium impudicum	Dinoflagellates	sPS	Mannose, galactose, glucose
Aphanothece halophytica	Cyanophytes	EPS	Glucose, fucose, arabinose, mannose

*sEPS = soluble Extracellular Polysaccharides, sPS = soluble Polysaccharides, PS = Polysaccharides, EPS = Extracellular Polysaccharides

2.12. Applications of polysaccharides

Seaweeds are considered industrially as a potential source of polysaccharides such as carrageenans (*Chondrus, Eucheuma* and *Kappaphycus*), alginates (*Ascophyllum, Laminaria*) and agars (*Gelidium and Gracilaria*) with a global value of around $ US 1 billion. These polysaccharides are widely used in the food (stabilisers, thickeners, and emulsifiers), textile, paint, biotechnological and biomedical industries (Mishra *et al.* 2011). Recently, polysaccharides have attained the spotlight as functional food ingredients. The majority of these species are used in some form for food and chemical extracts.

Polysaccharides in therapeutic applications

***Anti-viral properties*: ** Studies have reported that the extracellular secretions comprised of polysaccharide in the medium exhibit anti-viral activity against different kinds of viruses. As reported, anionic and sulphated polysaccharides from *Porphyridium* and *Arthrospira* exhibit anti-viral properties against Herpes simplex virus, and Varicella zoster virus (Challouf *et al.* 2011). PS from *A. plentesis*, termed TK-V3 is found to be active against *Vaccinia* and *Ectromelia orthopox* virus (Radonić *et al.* 2011). The degree of sulfation and the content of uronic acid of any EPS are found to be associated with the anti-viral activity (Damonte *et al.* 2004). Calcium spirullan (CaSP), another intracellular polysaccharide isolated from *A. platensis* is reported to inhibit the viral infection by inhibiting the penetration of virus into the host cell (Hayashi *et al.* 1996).

***Free radical scavenging activity*:** Photosynthetic algae due to their constant exposure with light generate free radicals which are overcome by the production of scavenging complexes. The major application of anti-oxidant properties of EPS lies in the prevention of lipids oxidation into the hydroxyl ions, hydrogen peroxide, and super oxide ion through reactive oxygen species in the pharmaceutical companies as oxidation of lipids degrades the quality of the nutritional products and the stability of the product. sPS from *Porphyridium* has shown the effective free radical scavenging activity against the oxidation of a particular fatty acid i.e. linoleic acid (Dvir *et al.* 2009). The polysaccharides associated with sulphur and proteins greatly enhance the activity in a dose dependent manner. EPS from *Porphyridium creuntum* and *Rhodella reticulata* has been observed to exhibit free radical scavenging activity as they were effectively scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Chen *et al.* 2010).

Anti-inflammatory and immunomodulatory activity: Polysaccharides from marine microalgae such as *Porphyridium*, *Phaeodactylum*, *C. stigmatophora* have been explored for

exhibiting various pharmacological properties such as anti-inflammatory, immunomodulatory activity. Besides the demonstration of anti-inflammatory activity shown by the EPS of *Porphyridium* and *C. stigmatophora*, certain polysaccharides from *P. tricornutum* and *C. stigmatophora* possess immunostimulatory activity and immunosuppressant activity respectively (Guzman *et al.* 2003). p-KG03(sPS) obtained from *Gellidium impudicum*, activates the production of nitric oxide and cytokines by the RAW 264.7 mouse macrophage cells (Bae *et al.* 2006). In contrast, polysaccharides from *Porphyridium* inhibit the adhesion of blood leukocytes which ultimately decrease the secretion of cytokines in serum. Likewise, sPS from *Porphyridium* species exhibited immunostimulatory activity by increasing spleenic and thymus index in mice suffering from S180 tumors (Sun *et al.* 2012).

Apart from showing the above described properties of polysaccharides from microalgae, they are also involved in following properties:

- Anti-tumour
- Anti-lipidemic and Anti-glycemic properties
- Anti-thrombotic and anti-coagulant properties
- Biolubricant properties
- Anti- adhesives
- Nutraceuticals
- Nanofibre production
- Drag reducing capacity
- Soil aggregation

Chapter III

Objectives

3. Objectives

1. Assessment of immunological effect of the isolated crude exopolysaccharides from *Dunaliella salina* on peripheral blood mononuclear cells and RAW 264.7 mouse macrophage cell lines
2. Determination of immunomodulation effect of high sugar containing fractions obtained by chromatography
3. Characterisation of the fractions by Mass spectroscopy and Fourier Transform infrared spectroscopy
4. Enhancement of sugar content by providing different nutritional and salt stress conditions

Chapter IV

Methods and Methodology

I. Materials

Table 4.1. List of reagents

	Reagents/ Chemical	Company
1	ABTS (2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonicacid]-diammonium salt) substrate	ThermoFisherScientific, USA
2	Amphotericin B	Sigma Aldrich, USA
3	Bovine serum albumin	Sigma Aldrich
4	Concanavalin A	Sigma Aldrich
5	Dimethyl Sulphoxide	Merck, Germany
6	Foetal bovine Serum	Gibco®Life Technologies, USA
7	Glutamine	Himedia, India
8	HEPES buffer	Sigma Aldrich
9	Histopaque® -1077	Sigma Aldrich
10	Human IFN-γ Mini ABTS ELISA Development Kit	PeproTech, USA
11	MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Sigma Aldrich
12	Penicillin Sodium	Himedia
13	Potassium Chloride (KCl)	Himedia
14	Potassium phosphate monobasic (KH_2PO_4)	Himedia
15	Recombinant human Interleukin-2	Sigma Aldrich
16	Rosewell Park Memorial Institute (RPMI)-1640 medium	Sigma Aldrich
17	Sodium Bicarbonate ($NaHCO_3$)	Himedia
18	Sodium Chloride (NaCl)	Himedia
19	Sodium phosphate dibasic (Na_2HPO_4)	Himedia
20	Streptomycin	Sigma Aldrich
21	Trehalose	Sigma Aldrich
22	Trypan blue	Himedia
23	Tween 20	Sigma Aldrich

II. Methodology:

4.1. ***Procurement of the algae***

The culture of microalgae *Dunaliella salina* (*D. salina*) was procured from Birla Institute of Scientific Research, Jaipur, India

4.2. Culture of microalgae

Growth parameters: Microalga *D. salina* was grown on AS100 media which composed of different salts as described in Table 1 (Starr 1987). Media was prepared according to the defined molarities and was autoclaved at 20 psi and holding time (t_h) =15 min.

KH_2PO_4 and $FeCl_3$ were autoclaved separately to avoid precipitation and filter sterilised $NaHCO_3$ was added in the medium. pH was finally adjusted to 7.5. 10 % of the medium with the cell density of 3-5×10^6 cells/mL was used as the volume for subculturing for the production of metabolites.

The culture was grown in 500 ml Erlenmeyer flask containing 100 ml medium. The initial 100 ml of the culture served as the mother culture. The working culture volume was kept 1000 ml. The culture was grown in controlled laboratory conditions i.e., 25 ± 2°C temperature and 12/12 h light and dark period for 20 days. The cultures were constantly shaken. Sub culturing was done after every 20 days (Mishra & Jha 2009).

4.3. Stress conditions

D. salina is known to survive in wide range of salt and nutrient concentrations although that puts the organism into stress (Mishra & Jha 2009). This leads to either increase or decrease in the production of the metabolites (β-carotene, glycerol and EPS). To enhance the production of EPS, two nutrients (KNO_3 and KH_2PO_4) and one salt stress (NaCl) were provided in two different concentrations (KNO_3 = 14.7 mM and 24.5 mM; KH_2PO_4 = 4.2 mM and 7 mM; NaCl = 3 M and 3.5 M).

Table 4.2: Chemical Composition of AS-100 medium

Salts	Concentration (molarity)
NaCl	1.7 M
$MgCl_2.6H_2O$	7.3 mM
$MgSO_4.7H_2O$	2.0 mM
KCl	2.6 mM
$CaCl_2$	2.3 mM
KNO_3	4.9 mM
Tris- buffer	4.9 mM
EDTA-di-Na salt	8.5 µM
$ZnCl_2$	0.58 µM
H_3BO_3	19 µM
$CoCl_2$	0.12 µM
$CuCl_2$	0.46 µM
$MnCl_2$	6.3 µM
$FeCl_3$	9.2 µM
KH_2PO_4	1.4 mM

4.4. Measurement of growth

4.4.1. *Cell count*

The growth of culture was measured in the terms of cell number which was counted every three days for 20 days using nauberg haemocytometer. As the cells of *D. salina* are motile in nature, the lugol's iodine solution was used to fix the cells for counting (Auinger *et al.* 2008).

4.4.2. *Specific growth rate*

Specific growth rate was calculated by measuring the absorbance at 560 nm after every three days by spectrophotometer. The specific growth µ (max) was calculated by the standard understated equation (García-González *et al.* 2005).

$$\mu\ (max) = 3.3(\log N - \text{Log } N_o) / t$$

Where,

µ (max) = specific growth

N = Final reading at 560 nm

N_o = Initial spectrophotometer reading at 560 nm

t = Time

4.5. Downstream processing of extracellular polymeric substances

4.5.1. Extraction

After 20 days of growth, the culture was centrifuged at 10000 rpm for 30 min to remove the debris and cells. The supernatant was collected, filtered with whattman filter paper and heated at 60-70°C till the volume reached the $1/5^{th}$ or $1/6^{th}$ of the original volume. The concentration procedure was followed by the organic precipitation. Equal volume of cold methanol was used for the precipitation of extracellular polymeric substances in the concentrated culture and kept for 16-18 h in 4°C. It was followed by centrifugation at 10000 rpm for 10 min to remove methanol and the pellet was dissolved in MQ (Milli Q) water after washing it twice with absolute ethanol. The dissolved pellets were heated to dissolve any clumps and filtered (Mishra & Jha 2009).

4.5.2. *Dialysis*

The dissolved pellet was dialysed to remove salts against double distilled water for 48 h. Water was changed three times a day. Dialysis membrane tubings were activated beforehand (Marusyk & Sergeant 1980).

***Dialysis membrane activation and tubing preparation*:** The dialysis membrane of pore size 12 kDa was purchased and tubing of 7-8 cm was cut. The tubings were immersed in 2% sodium bicarbonate solution and boiled for 10 min. The solution was discarded and again boiled in distilled water for 10 min. Water was discarded again and the activated membranes were stored in 50% ethanol at 4°C.

4.5.3. *TCA precipitation*

The dialysed liquid was then subjected to TCA (tri chloro-acetic acid) precipitation to remove proteins from the extracted sample (Link & LaBaer 2011). For TCA precipitation, 100% TCA was made as stock. The working concentration of 20% was prepared freshly. Then, TCA was added to the dialysed liquid and incubated for at least 1h. Then, it was centrifuged at the maximum speed (14000 rpm) for 10 min. The pellet of proteins was discarded.

4.5.4. *Lyophilisation*

TCA precipitated dialysed liquid was lyophilised. The samples were frozen to -80ºC beforehand. Then, the process of lyophilisation was carried out at -60ºC to get powder (Coppa *et al.* 1999).

4.6. Estimation of biomolecules in extracellular polymeric substances

4.6.1. ***Estimation of polysaccharide content***

The polysaccharide content was estimated by phenol sulphuric assay (Masuko *et al.* 2005). Sulphuric acid hydrolyses polysaccharides in the monosaccharide units and expose the reducing ends of even non-reducing ends. The dehydrated monosaccharide units thus react with phenol to the development of yellow golden colour. Sample (250 µg/mL) was added in each well and water was used for volume makeup to 50 µL. Similarly, glucose standards were prepared in the range of 10-150 µg/mL in water. Following this step, 150 µL sulphuric acid was added rapidly. Immediately, 30 µL of 5% phenol prepared in distilled water was added. The microplate was incubated in water bath at 70°C for 10 min. The plate was brought to the room temperature and the absorbance was observed at 492 nm. The amount of glucose equivalents in the sample was calculated by the standard curve equation obtained.

4.6.2. ***Estimation of total protein content***

Bradford assay is a rapid and most sensitive assay for protein estimation (Bradford 1976). Coomassie brilliant blue G250 dye is used to detect proteins as this dye binds to the certain amino acids and thus leads to the formation of a complex whose extinction co-efficient or the molar absorptivity increases from 465 to 595 nm. This is why; the absorption at 595 nm is measured. The assay is useful as the time taken to complete this assay is less than 2 min. The interfence may be caused due to strong alkaline buffering agents. Care must be taken during handling as commonly used detergents such as triton 100 or SDS could interfere in the absorbance.

Bradford reagent preparation*:* 100 mg of coomassie brilliant blue dye G250 was dissolved in 50 mL of 95 % ethanol. Following this, 100 mL of 85% phosphoric acid was added and the volume was made up to 600 mL with distilled water (D.W.) then, the solution was filtered and 100 mL of glycerol was added and the volume was made to 1000 mL. The resultant solution was used after 24 h. The prepared reagent should be stored in detergent free bottle in 4ºC

Procedure for estimation: The test was done in 96 well microtitre plate. For estimation, 250 µg/mL of the sample was added in the well. Likewise, the bovine serum albumin (BSA) standard was prepared in the range of 10-100 µg/mL. This step was followed by the addition of 100 µL of prepared Bradford reagent. Total volume was kept 150 µL and

the solution was incubated for 5 min at room temperature. The absorbance was observed at 595 nm. The sensitivity of this assay is 1-20 µg.

4.6.3. Nucleic acid assessment in extracted EPS

Nucleic acids i.e. DNA and RNA contamination was assessed by spectrophotometric method (Wilfinger *et al.* 1997). The absorbance of the extracted EPS was observed at 260 and 280 nm and then the ratio was calculated. The ratio of 1.8 confirms the presence of nucleic acid and ratio higher than 2 confirms the presence of proteins.

4.6.4. Assessment of different phytochemicals

Qualitative phytochemical tests were performed to check the presence of alkaloids (Wagner's test), flavanoids (Alkaline reagent test), phenol (Ferric chloride test), tannins (Braymer's test), terpenoids (Salkowski's test) and quinones (conc. HCl test) as per standard procedure (Aziz 2015).

Ferric chloride test for phenols: 20 µL polymeric extract was treated with aq. 5% ferric chloride solution and observed for the formation of deep blue/ black colour.

Braymer's test for tannin: The polymeric extract was treated with 10% of alcoholic ferric chloride solution. Then, it was observed for the formation of blue and greenish colour solution.

Wagner's test for alkaloids: The polymeric extract was treated with 3-5 drops of Wagner's reagent (1.27 g of iodine +2 g of potassium iodide in 100 mL of water). The presence of alkaloids was observed by the presence of reddish brown precipitate.

Salkowaski's test for terpenoids: 100 µL of methanol was added to the polymeric extract. Few drops of concentrated sulphuric acid (H_2SO_4) were added to the solution and reddish-brown precipitate was observed immediately for the presence of terpenoids.

Concentrated HCl test for quinones: The polymeric extracts were treated with drops of concentrated HCl and were observed for the presence of yellow coloured precipitate.

Alkaline reagent test for flavanoids: 100 µL of the extract was treated with few drops of 20% sodium hydroxide and it was observed for the presence of deep yellow colour.

4.7. Maintenance and handling of cell lines

4.7.1. Preparation of media

Dulbecco's modified eagle's media (DMEM) was used for the culturing of RAW 264.7 cell line and RPMI 1640 for culturing of peripheral blood mono-nuclear cells. DMEM is a modification of Basal Medium Eagle (BME) that contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary components whereas RPMI 1640 is best suited for the suspension cultures as it contain

high amount of vitamins (biotin, vitamin B12, inositol, choline) and glutathione which are lacking in DMEM. For the present work, DMEM and RPMI 1640 media which is suitable for autoclaving is used.

Media was prepared according to the manufacturer's instructions. Briefly, 9.6 g of powdered media was dissolved in 900 mL of MQ water with constant and gentle stirring. pH of the media was adjusted to 4 before the addition of sodium bicarbonate and glutamine solution. 26.5 mL of 4% sodium bicarbonate solution and 20 mL of 200 mM glutamine solution was added to the media. The final pH of the media was adjusted to 7.4 with the help of 1N HCl and 1N NaOH solution. Further, 10 mL of antibiotics solution (100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin) was also added to the media. Media was adjusted to the volume of 1000 mL with MQ water. Then, media was subjected to filtration through 0.22 µm filter membrane under vacuum conditions. The sterile media was stored in -20ºC in dark till use. RPMI 1640 was also prepared in the same way as DMEM media.

4.7.2. Preparation of phosphate buffer saline

One litre of 1x Phosphate buffer saline (PBS) was prepared by adding 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g of KH_2PO_4 in 900 mL of MQ water. The pH was adjusted to 7.4 with the help of 1N HCl and 1N NaOH solution. Then, the volume was increased to 1000 mL and then was autoclaved with holding time of 15 min at 15 psi and 121ºC. The buffer was then stored at 4ºC.

4.7.3. Revival of cell lines

The frozen cell lines in the vials stored at -80ºC were thawed rapidly till complete melting and suspended in 8 mL of complete growth media (DMEM supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin and 10 % FBS). The falcon was centrifuged at 2000 rpm for 10 min. The pellet was washed with 5 mL of 1x PBS buffer. The pellet obtained was suspended in 1 mL of complete DMEM media. Then, the counting was done using haemocytometer using trypan blue exclusion assay. The cells were seeded in T25 cell culture flask supplemented with 5 mL of complete growth media and incubated in 37ºC with 5% CO_2 in CO_2 incubator. Flask was monitored regularly for its growth, contamination and pH change.

4.7.4. Freezing of the cell lines

The repeated sub culturing of cells leads to the accumulation of changes and the cell population no longer remains with the original morphological and metabolic properties. It

is believed that cell loses its properties after 80-100 sub culturing. In order to prevent this effect, cells are freezed at very low temperatures to seize their metabolic activity.

Briefly, the procedure of freezing begins with the trypsinisation of the 80% confluent flask. The pellet obtained after trypsinisation was dissolved in 1 mL complete media to count the cells with the help of haemocytometer. Then the dissolved cells were centrifuged again at 2000 rpm for 10 min to obtain the pellet. The pellet was dissolved in 80% FBS, 10% DMEM complete media, 10% DMSO to make up the volume to 1 mL in a centrifuge tube. The centrifuge tube was then kept at 4ºC for 1h followed by 0ºC for 2h. Then, it was transferred to -20ºC for 2-4 h followed by shifting it to -80ºC.

4.7.5. Maintenance of cell lines

The anchorage dependent cells grow in monolayers as they communicate with each other through paracrine signalling. Complete DMEM media was used for cell culture maintenance and was changed after every two days. For media change, firstly the old media was discarded from the culture flask. Then, the flask was washed with 3 mL of 1x PBS. Tapping was done to remove cell debris and then, fresh complete media was added to the flask. After the attainment of 70-80% confluency, sub culturing was done. Warm trypsinisation method was used for sub culturing. Trypsin is the protease which cleaves the integrins to detach the adherent cells from the collagen coated culture flask. For the process of trypsinisation, firstly, old media was decanted from the flask and washed with 1x PBS. 3 mL of 1x trypsin solution was added to the flask and then incubated at 37ºC in CO_2 incubator for 5 min. After 5 min, equal volume of complete media was added to stop the reaction of trypsin. The solution was centrifuged at 2000 rpm for 10 min. The pellet was resuspended in 1 mL of complete media and counting is done by trypan blue dye exclusion method (Strober 2001). The cells were divided into appropriate split ratio and seeded in either T25 or T75 flask. The cells were regularly monitored for morphology, pH change, contamination and growth.

4.7.6. Cell enumeration

Cell counting was done with the help of haemocytometer using trypan blue exclusion assay (Strober 2001). It works on the principle that dead cell accumulates the dye and appears blue whereas living cell appears shiny. To prepare the slide, 10 µL of cell suspension was diluted with 0.4% trypan blue dye (prepared in 1X PBS) in either 1:5 or 1:10 ratio. The cells were observed under light microscope under 40x magnification (Nikon Eclipse E100 LED). Cells were counted in all four squares and the calculations were made according to the standard equation (Figure 4.1).

$$A = (Y \times X \times 10^4) / 4$$

Where,

A = Number of viable cells per mL

Y = Number of cells counted in the four squares of haemocytometer

X= dilution factor

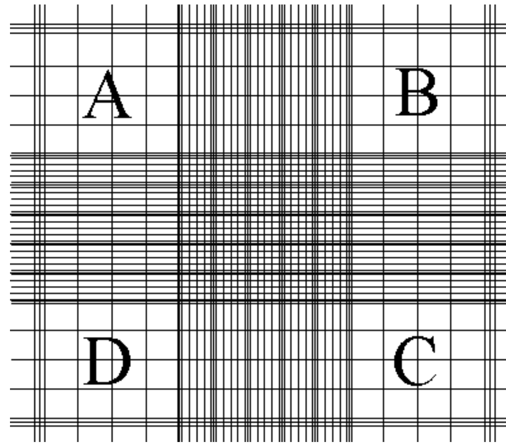

Figure 4.1: Representation of haemocytometer. A, B, C and D represents the four chambers which are taken into account in order to count the cells

4.7.7. Isolation of peripheral blood mononuclear cells (PBMC)

Blood (5 mL) of the healthy donors was drawn via veni puncture by the trained technicians from Lifeline blood bank, Patiala and Rajendra Hospital, Patiala in blood collection EDTA coated tubes (BD vacutainer ® Tubes). The informed consent was given by all the donors for the experiments. The study has got the approval from the institutional ethical committee (IEC).

PBMC were isolated by ficoll density gradient method (English & Andersen 1974). Blood was layered carefully onto histopaque 1577 in 1:1 ratio in a 15 mL falcon. It was followed by centrifugation at 450 g for 30 min at room temperature in swinging bucket rotor (Thermo scientific Biofuge Stratos). After centrifugation, plasma was discarded and buffy coat containing PBMC was collected very carefully. Cells were washed twice with 8 mL of 1x PBS and centrifugation was done at 250 g for 10 min. The pellet was then resuspended in 1ml complete RPMI 1640 media containing 10% (v/v) FBS , 100 IU/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin.

4.8. Assessment of cell growth by MTT assay

The effect of EPS on PBMC cell proliferation was assessed by MTT (3- 4, 5 dimethylthiazolyl-2-2-5, diphenyltetrazolium) based calorimetric assay (Denizot & Lang 1986). The assay is based on the principle where the MTT reduces to a purple coloured product called formazan due to the presence of succinate dehydrogenases enzymes secreted from mitochondria of the metabolic active eukaryotic cells (Figure 4.2) (van Meerloo *et al.* 2011). The product is produced in the form of crystals which are required to be dissolved by dimethyl sulfoxide (DMSO). Another method which is similar to MTT is XTT method where the reduced product formed is already in dissolved form. Originally, MTT assay was used to determine cell proliferation but indirectly, if cells undergo necrosis or apoptosis, the reduction in cell viability can also be measured. For each reaction, the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

MTT assay on PBMC

Freshly isolated PBMC cells were seeded in 96 well microtitre plate at the density of 2×10^5 / well. EPS were added in the wells in the concentration varying from 250-1500 µg/mL. The experiments were performed in triplicates. Three independent experiments were designed to confirm the results. The volume was made up by complete media (RPMI 1640) to 200µl. 10 µg/mL Concanavalin A (ConA) served as the positive control. After 48h of incubation at 37ºC in CO_2 incubator with 5% CO_2, 20 µL MTT (5 mg/mL in PBS) was added in each well. Following the addition, the plate was again incubated for 4h. The plate was then centrifuged for 10 min at 2000 rpm to settle down the crystals. 170 µL of media was discarded and 100 µL of DMSO was added in each well. The absorbance was recorded at 570nm taking reference wavelength at 620nm on ELISA plate reader (Tecan Infinite Pro ELISA reader).

MTT assay on RAW 264.7 cell lines

Prior to the test, the culture flask with 70-80% confluency was trypsinised and cells were counted. Then, the cell culture microtitre plate was seeded at the density of 10^4 cells per well. The volume was made up to 200 µL by complete media (DMEM). The seeding was followed by overnight incubation before adding the sample. 2 µg/mL LPS (lipopolysaccharide) served as positive control. After 48h of incubation at 37ºC in CO_2 Incubator with 5% CO_2, 20 µL MTT was added in each well. Following the addition, the plate was again incubated for 4h. 170 µL of media was discarded and 100 µL of DMSO

was added in each well. The absorbance was recorded at 570nm taking reference wavelength at 620nm on ELISA plate reader (Tecan Infinite Pro ELISA reader).

The cell growth was calculated as the function of cell viability percentage with the help of equation given below:

$$\text{Proliferation index} = (A / B)$$

Where,

A= Absorbance of sample (cells treated with EPS)

B= Absorbance of blank (cells only)

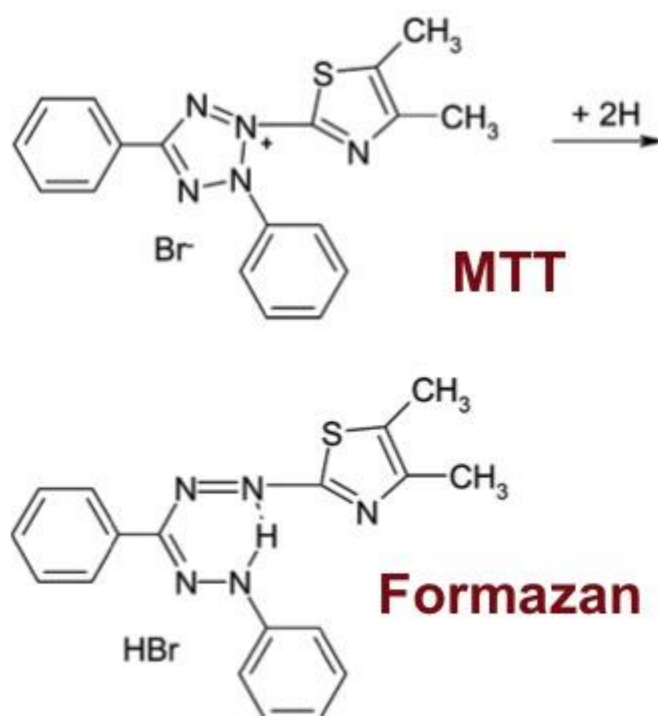

Figure 4.2: Principle of MTT assay

4.9. Nitric Oxide Estimation Assay

The release of nitric oxide (NO) was measured in mouse macrophage cell line (RAW 264.7) with the help of griess reagent (Pandey *et al.* 2005). Cells were seeded at the density of 2.5×10^5 cells/well and incubated overnight for attachment. Next day, media was changed and sample in the concentration range of 250 - 1500 µg/mL along with positive control (10 µg/mL LPS) was added in the well. The experiment was performed in triplicates. The sample addition was followed by 48h of incubation at 37ºC in presence of 5 % CO_2 in CO_2 incubator. NO production in the supernatant was measured in terms of nitrite production which is the stable product. 100 µL of the supernatant was aspirated and mixed with 100 µL of griess reagent (1% sulphanilamide in 5% H_3PO_4 and 0.1% N-

napthyl ethylene-diamine-di-hydrochloride in 5% H_3PO_4 (Sigma)) at room temperature for 10 min. The absorbance was recorded at 570nm.

4.10. Assessment of anti-oxidant effects of EPS

In order to analyse the anti-oxidant capacity of exopolysaccharides, DPPH assay was performed (Mishra *et al.* 2012). DPPH gets reduced when hydrogen donor is present in the reaction mixture and a colorimetric change from violet to pale yellow colour is observed due to the formation of 2, 2-diphenyl-1-picrylhydrazine (Figure 4.3).

Briefly, EPS were prepared in water in the concentration range of 250 to 1500 µg/mL in the 96 well plate. 30 µL Ascorbic acid (100µM) prepared in distilled water was taken as the standard. 150 µL of DPPH prepared in methanol was added to each well. A blank of distilled water was also run simultaneously with the test. The plate was incubated in dark for 45 min and the absorbance was measured at 517nm. Scavenging activity was calculated according to the following equation:

Radical scavenging activity (%) = (Control $_{abs}$ – Sample $_{abs}$ / Control $_{abs}$) × 100

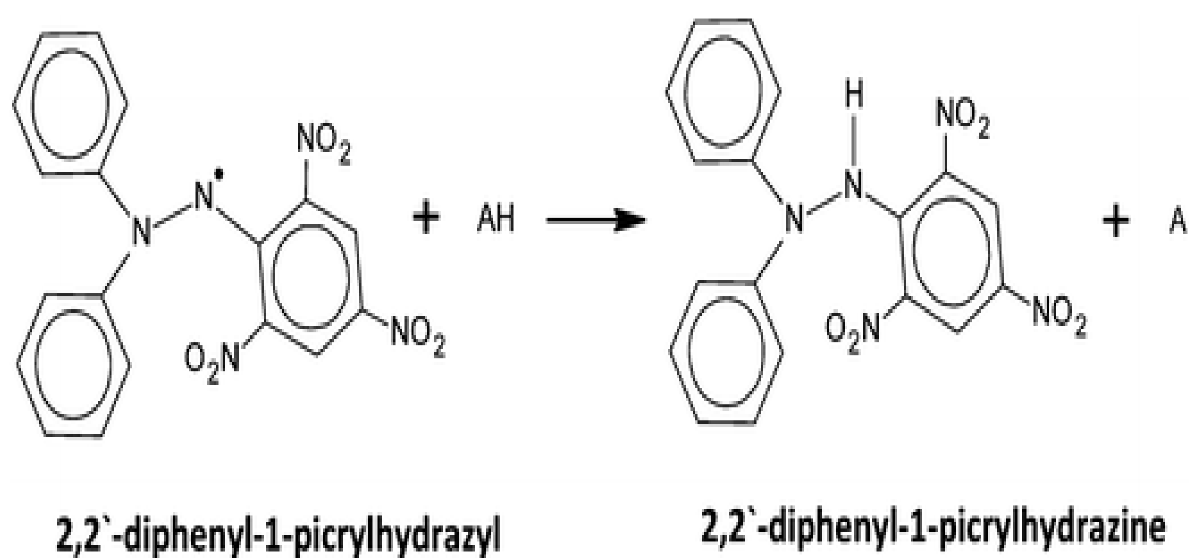

Figure 4.3: Working of DPPH assay (Mishra *et al.* 2012)

4.11. Cytokine estimation

PBMC were cultured in the density of 2.5×10^5 cells/well and samples were added for their analysis in varying concentration (250-1500 µg/mL). After the incubation of 48h at 37ºC in CO_2 incubator with 5% CO_2, 100 µL supernatant was collected in the micro-centrifuge tubes for analysis of three different cytokines (TGF-β, IFN-γ, TNF-α). The supernatants can be stored in -20ºC.

IFN-γ

IFN-γ secretion in the culture supernatant was measured by sandwich ELISA (L. Darwich*et al*., 2008) in 96-well ELISA plate as per manufacturer's instructions. 100 µl of capture antibody (1µg/mL in PBS) was added to each ELISA plate well and incubated overnight at room temperature. The wells were aspirated to remove liquid and the plate was washed four times with 300 µL wash buffer (0.05% Tween-20 in PBS) per well. After the last wash, the plate was tapped in inverted position to remove residual buffer on paper towel. 300 µL of blocking buffer (1% BSA in PBS) was added to each well and the plate was incubated for 1 h at room temperature. After washing the plate four times with wash buffer, 100 µL of the test sample and the IFN-γ standard were added to each well in triplicate. The plate was incubated at room temperature for four hours or overnight. The plate was washed 4 times and 100 µL of detection antibody (1µg/mL in sample diluent buffer) was added to each ELISA plate well and incubated at room temperature for 2 h. Plate was washed four times and 100 µL of diluted avidin-HRP conjugate (1:2000) in sample diluent was added and incubated for 1 h. Plate was again washed four times and 100 µL of ABTS substrate solution was added to each well. The plate was wrapped in a foil and incubated at room temperature for 15 min for colour development. Absorbance was recorded at 405 nm with wavelength correction set at 650 nm in ELISA plate reader (Tecan, Austria).

TNF-α

Similarly, TNF-α secretion in the culture supernatant was also measured by sandwich ELISA (L. Darwich*et al*., 2008) in 96-well ELISA plate as per manufacturer's instruction. Firstly, ELISA plate was incubated overnight with 100 µL of capture antibody (1 µg/mL in PBS) at room temperature. The wells were aspirated to remove liquid and the plate was washed four times with 300 µL wash buffer (0.05% Tween-20 in PBS) per well. After the last wash, the plate was tapped in inverted position to remove residual buffer on paper towel. 300 µL of blocking buffer (1% BSA in PBS) was added to each well and the plate was incubated for 1 h at room temperature. After washing the plate four times with wash buffer, 100 µL of the test sample and the TNF-α standard were added to each well in triplicate. The plate was incubated at room temperature for four hours or overnight. The plate was washed 4 times and 100 µL of detection antibody (1 µg/mL in sample diluent buffer) was added to each ELISA plate well and incubated at room temperature for 2 h. Plate was washed four times and 100 µL of diluted avidin-HRP conjugate (1:2000) in

sample diluent was added and incubated for 1 h. Plate was again washed four times and 100 μL of ABTS substrate solution was added to each well. The plate was wrapped in a foil and incubated at room temperature for 15 min for colour development. Absorbance was recorded at 405 nm with wavelength correction set at 650 nm in ELISA plate reader (Tecan, Austria).

TGF-β

Likewise, TGF-β secretion was also measured by sandwich ELISA. Although the procedure was similar as was for IFN-γ and TNF-α but here, capture antibody was already coated onto the ELISA plate available in the kit. The samples along with the standards were coated onto the plate and incubated overnight. Then, the plate was washed three times with the 1x washing buffer (diluted from 20x) available in the kit. After the last wash, the plate was tapped in inverted position to remove residual buffer on paper towel. 300 μL blocking buffer (also available in the kit) was added to the wells and incubated for 1 h. The plate was again washed with 1x washing buffer thrice. 200 μL of detection antibody (diluted 500x) was added in the plate and incubated for 2 h. Again, the plate was washed with 1x washing buffer. It was followed by the addition of HRP conjugate (diluted 80x) incubated for 2 h. Again it was washed thrice with 1x washing buffer. Then, the TMB substrate available in the kit was added in the each well and incubated again for 30 min. The absorbance was recorded at 450 nm after adding 50 μL stop solution in each well.

4.12. Thin Layer chromatography

Thin layer chromatography (TLC) is the method of analysing the solution of mixture of different compounds (Randerath & Libman 1966). It can be used to determine the number of compounds in a solution, purity of the compound or the identity of compound. It can also be used to monitor the progress of any reaction. The advantage of TLC is that the technique is very sensitive and is less time consuming. TLC is comprised of three steps; spotting, development and visualisation. Spotting refers to the transfer of small amount of sample on the starting end of the TLC plate (silica gel coated onto the aluminium sheet). It can be done either by the use of pipette or via a capillary. Spotting is followed by the development which is performed by placing the silica gel plate in a shallow pool of the solvent (mobile phase) which moves up the plate via capillary action. The compounds in the mixture move along with the solvent based on the polarity. Different solvents (20% DCM: Methanol, 50% DCM: methanol and 100% methanol) based on increasing polarity were tested to observe the maximum separation. Next step is visualisation which involves

the detection of various compounds which may be present in the mixture of the solution. As most of the compounds are colourless, this is very important step. Firstly, the developed plate was observed in UV light to look for the presence of UV active compounds. Then, to check the presence of other compounds, the plate was treated with four different (p-anisaldehyde, ninhydrin, 2,4-DNP, $KMnO_4$) detection stains(Randerath & Libman 1966).

4.12.1. Preparation of detection stains

P- anisaldehyde: This stain is for detecting the groups with nucleophilic properties. For preparation, 15 ml of acetic acid and 3.5 mL of p-anisaldehyde were added to 350 mL of ice cold ethanol (Wagner & Bladt 1996). Then, 50 mL of concentrated sulphuric acid was added dropwise over 60 min. The unused portion was stored at 0ºC.

Ninhydrin: The stain is for the detection of amino acids. 1.5g ninhydrin was dissolved in 100 mL of n-butanol. Then, 3 mL of acetic acid was added.

$KMnO_4$: The stain is to detect olefins and other readily oxidised groups. It was prepared by dissolving 1.5g potassium per magnate, 10g potassium dichromate and 1.25 mL of 10% sodium hydroxide solution in 200 mL of water.

2, 4- DNP: The stain is to detect aldehydes and ketones in any sample. To prepare the stain, 12g of 2, 4-dinitrophenylhydrazine, 60 mL of sulphuric acid and 80 mL of water was dissolved in 200 ml of ethanol.

4.13. Fractionation of the crude EPS via chromatography

Column preparation: column was packed with the slurry of silica gel in hexane. It was packed till 3/4th of the column length. Hexane was run along the column for packing. (*Note: column should never be dried)

Sample preparation: Initially, 400mg powder to be separated was put in a small round bottom flask. Then, slurry was made with ethyl acetate and silica in minimum amount just for the adsorption of extracted crude sample. Then, the solvent was dried completely (* add more silica to dry). The dried powder was loaded in the column.

Elution: After loading the sample, 100ml of ethyl acetate solvent was run through the column and fractions were collected in test tubes. Then, to increase the polarity, 20% methanol: DCM was run and collected in test tubes. Elution was followed by further

increasing the polarity of the solvent to 50% methanol: DCM and finally by 100 % methanol.

4.14. Mass spectroscopy

Mass spectroscopy is the analytical technique which ionises the chemical compounds and the ions are sorted on the basis of mass to charge ratio(Herbert & Johnstone 2002). The spectra obtained measures the mass of the compounds present in any sample. The spectra is used to analyse the isotopic signature of any chemical compound, molecular masses as well as the structure elucidation of various compounds present in any sample. The sample (solid, liquid or gas) is ionized by bombarding with electrons to break the compounds into charged ions. The ions are then detected and arranged according to their mass/charge ratio in accordance with the abundance. The instrument is composed of three main components; ion source, analyser and a detector system (Figure 4.4). Mass spectroscopy has gained importance in analysing the compounds such as oligosaccharides, lipids, proteins and nucleic acids.

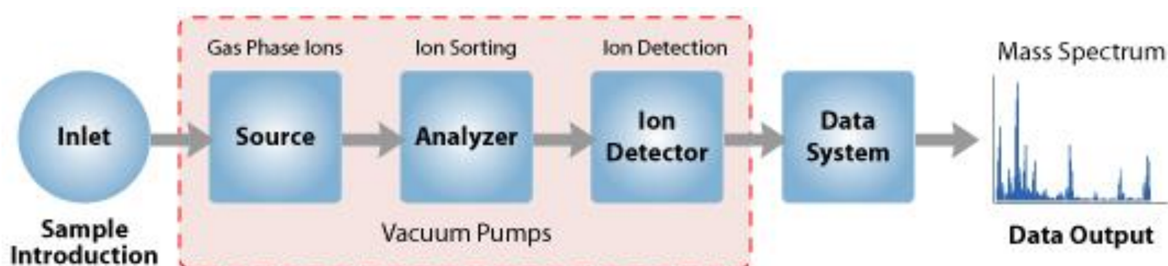

Figure 4.4: The components of a mass spectrometer

(Ref: http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html)

In the present study, mass spectroscopy for DsF1 and DsF4 was carried out in SAIF labs, Punjab University, Chandigarh. The details of the used technique are given in Table 4.3 and 4.4.

Table 4.3: Details of the instrument used

Instrument	Waters, Micromass Q-TOF micro
Separation Module	Waters Alliance 2795
Ionization	Electro spray Positive (ES+)
Acquisition	MRM, unit resolution

Injection Volume	20 micro litres
Flow rate	0.4 ml/min

Table 4.4: Parameters used of performing mass spectroscopy

Desolvation Gas	550 L/h
Cone Gas	30 L/h
Desolvation Temperature	300 ºC
Source Temperature	110 ºC
Capillary Voltage	3000V
Cone Voltage	30V
Collision energy	4ev
Gases used	N_2 and Ar
Mobile Phase used	20% H_2O and 80% Methanol
N2 supply pressure	6-7 bar(90-100psi)
Argon	5-6 Bar

4.15. Fourier transform infrared spectroscopy

FTIR expanded as Fourier Transform Infrared Spectroscopy is the method of passing infrared rays through the sample and the absorbance and transmittance patterns of any compound is imprinted (Nicolet & All 2001). Each molecule has its own pattern of arrangements of the chemical bonds having specific vibrational energies. These molecular bonds absorb light at particular wavelength and transmit light at different wavelength which results in the generation of spectra where peaks at particular wavelength corresponds to a particular chemical bond and the length of peak corresponds to the abundance. The spectrum obtained is unique to every compound as no two molecules can share the exact same infrared spectrum. Thus, this technique forms very important in identification of the particular compound. Basic instrumentation flow in FTIR includes a

source, interferometer, sample, detector and computer (Figure 4.5). Briefly, the infra-red light is emitted from a source which after passing through interferometer, passes through the sample and the transmittance is measured by the detector. The signals produced are input to a computer where fourier transformation is applied onto the spectra and the actual spectrum is obtained which is readable by the user.

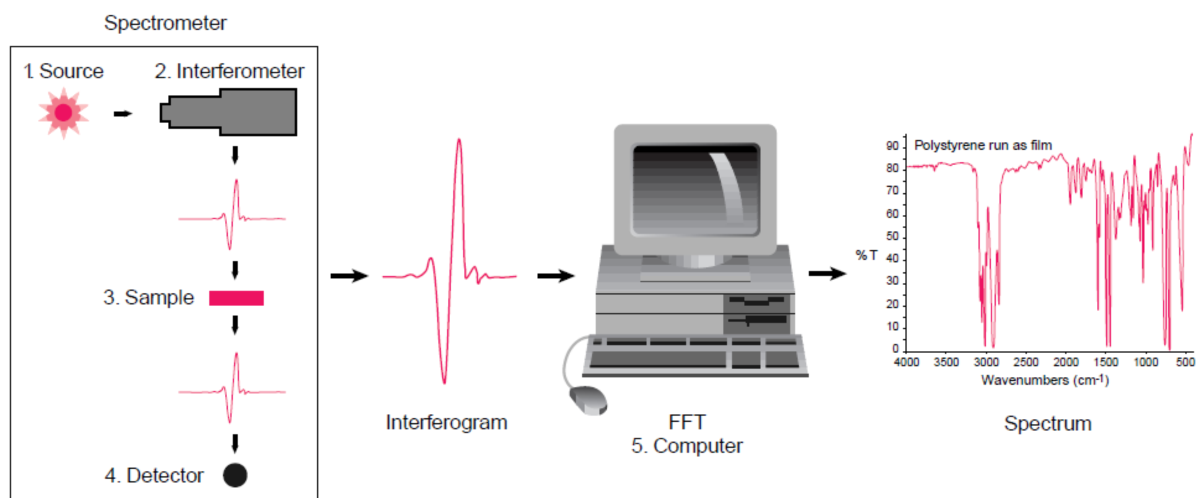

Figure 4.5: Flow of working of fourier transform infrared spectroscopy

Source: Introduction to Fourier Transform Infrared Spectrometery, Thermo Nicolet Corporation, 2001©

In the current study, FTIR analysis was performed with the help of Dr.Satyendra Pandey from the Department of chemistry, School of basic and applied sciences, Shri Guru Granth Sahib world University, Fatehgarh Sahib, Chandigarh, India.

4.16. Statistical analysis

The data were expressed as the mean ± standard error of mean of three independent experiments. Data were analysed using analysis of variance (ANOVA) and the means were compared using Tukey's test at $p < 0.05$.

Chapter V

Results

5. Results:

Objective1: Assessment of immunological effect of the isolated crude exopolysaccharides from Dunaliella salina on peripheral blood mononuclear cells and RAW 264.7 mouse macrophage cell lines

5.1. Biomolecules content in extracellular polymeric substances

The culture of *D. salina* was grown and monitored for 20 days followed by the extraction of extracellular substances. The extracted liquid was lyophilised to obtain the powder which was assessed for the presence of polysaccharides by phenol sulphuric assay taking glucose as a standard. Results concluded the presence of sugar in the extracellular secretion (Table 5.1).

The extracellular secretion was also assessed for the presence of proteins, nucleic acids and different phytochemicals. Due to the TCA precipitation, no proteins were detected when assayed through Bradford method keeping BSA as standard. Spectrophotometric analysis (A260/A280) confirmed the absence of any nucleic acids in the extracellular secretion.

Different phytochemicals i.e. flavanoids, alkaloids, tannins, cardiac glycosides and quinones were qualitatively assessed through the standard procedure and it was revealed that there were no phytochemicals present in the extracellular secretion.

Table 5.1: Sugar content in the extracellular polysaccharides (EPS)

Sample	Glucose equivalents(µg) /EPS (mg)
Crude	30.1 ± 5.6

5.2. Effect of crude extracellular polysaccharides (EPS) on peripheral blood mononuclear cells and RAW 264.7 cell lines

Crude EPS were assessed for their effect on peripheral blood mononuclear cells and RAW 264.7 mouse macrophage cell lines using MTT assay in the concentration range of 250-1500 µg/mL. It was observed that the proliferation index of PBMC decreased with increase in concentration (Figure 5.1). EPS concentration of 250 and 500µg/mL showed proliferation index more than 1 which shows enhanced cell proliferation of PBMC (Table 5.2). However in case of RAW cell lines, except the concentration of 250 µg/mL, the proliferation index is less than 1 indicating cell growth inhibition (Figure 5.2, Table 5.3). Along with the cell

viability assay, nitric oxide production by RAW macrophage cells was also measured through griess reagent. It was observed that nitric oxide production increased with concentration (Figure 5.3, Table 5.4). At the higher concentration of EPS (> 1000 µg/ml), the nitric oxide production is more than blank (cells only). The pattern of cell growth effect and NO production was found to be variable at different concentrations of EPS which may be due to presence of different compounds. Hence, the crude EPS was further fractionated by chromatographic techniques.

Table 5.2: The effect of crude EPS on the growth of PBMC

	Proliferation index
ConA* (5 µg /mL)	3.13 ± 18.1
EPS (µg /mL)	
250	2.25 ± 8.2
500	1.48 ± 5.5
1000	7.68 ± 6.1
1500	7.88 ± 4.6

*Con A = Concanavalin A

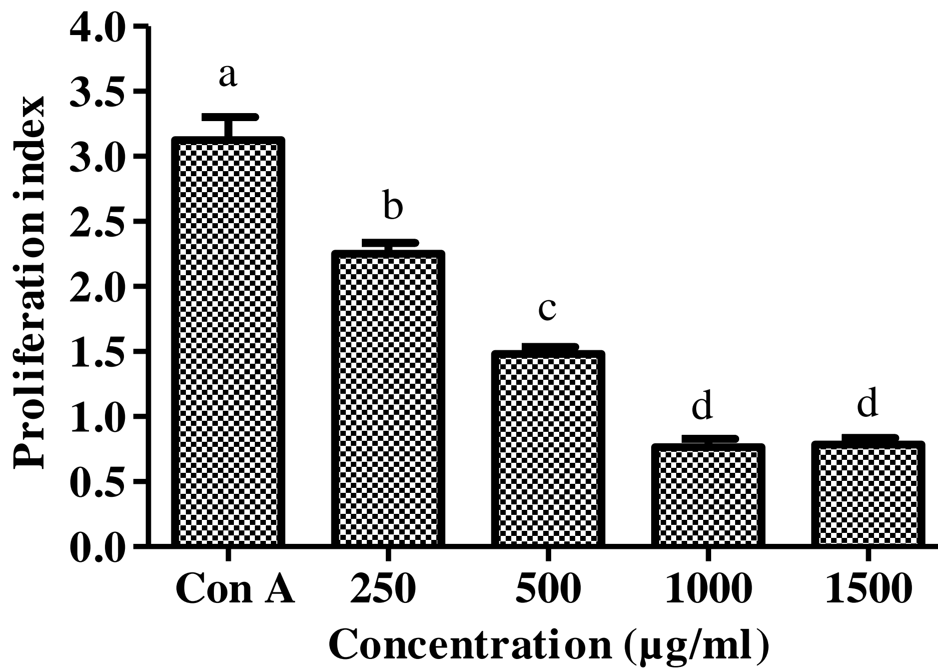

Figure5.1: Effect of crude EPS on the growth of peripheral blood mononuclear cells. Bars with the same lowercase letters are not significantly different at p>0.05. ConA: Concanavalin A (5 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the Con A/EPS treated cell and the untreated cells (Cells only).

Table 5.3: The effect of crude EPS on the growth of RAW 264.7 cell lines

	Proliferation index
LPS* (2 µg /mL)	1.61 ± 14.6
EPS# (µg /mL)	
250	1.154 ± 8.4
500	0.96 ± 1.7
1000	0.67 ± 8.8
1500	0.56 ± 3.3

*LPS = Lipopolysaccharide

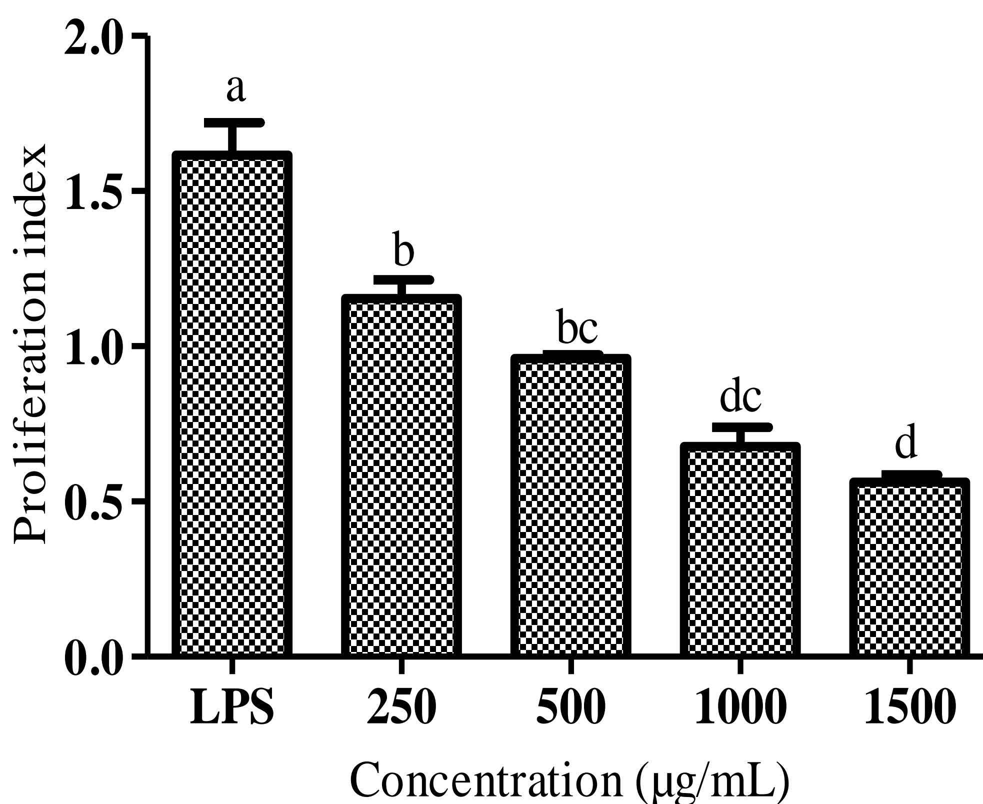

Figure 5.2: Effect of EPS on the growth of RAW 264.7 mouse macrophage cell lines. Bars with the same letters are not significantly different at p>0.05. LPS: Lipopolysaccharide (2 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the LPS/EPS treated cell and the untreated cells (Cells only).

Table 5.4: The effect of crude EPS on the release of nitric oxide from RAW 264.7 cell lines

Sample (µg /mL)	Absorbance (560nm)
Blank#	0.064 ± 0.004
LPS* (10)	0.086 ±0.001
EPS (250)	0.060 ± 0.001
EPS (500)	0.069 ± 0.001
EPS (1000)	0.105 ± 0.002
EPS (1500)	0.104 ± 0.008

*LPS = Lipopolysaccharide, #Blank = Cells only

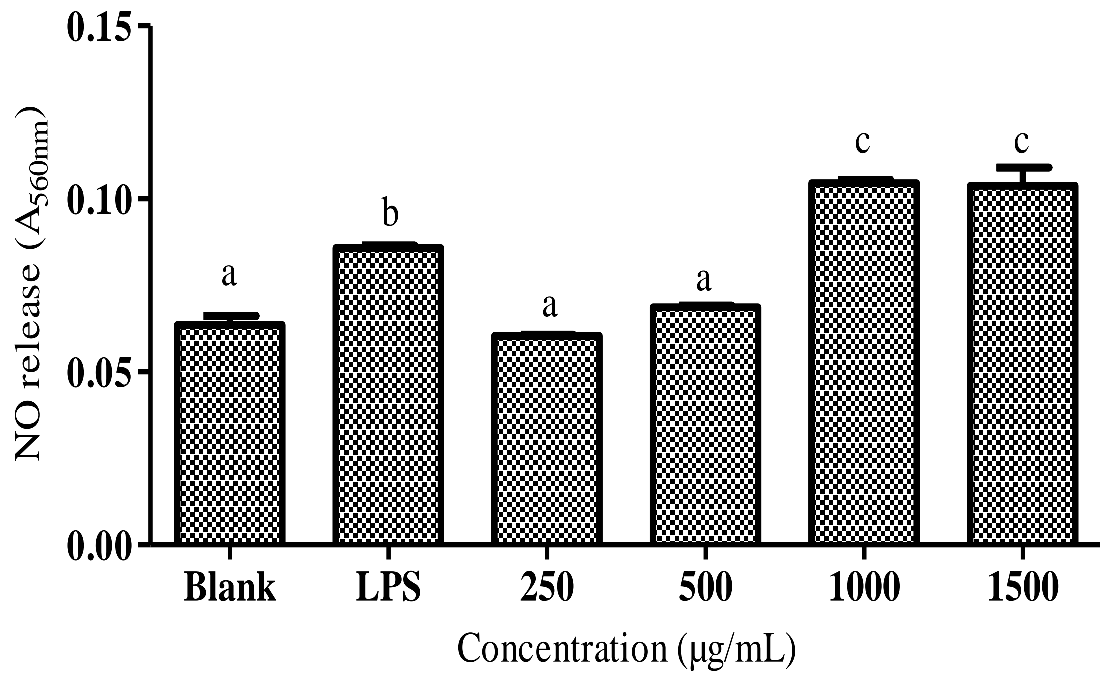

Figure 5.3: Effects of EPS on the production of nitric oxide from RAW 264.7 mouse macrophage cell lines. Bars with the same lowercase letters are not significantly different at $p>0.05$. Lipopolysaccharide (10 µg/mL) was used as a positive control. Blank: Cells only

Objective 2: Determination of immunomodulation effect of high sugar containing fractions obtained by chromatography

5.3. TLC analysis

The solubility of crude EPS was examined in four organic solvents of increasing polarity, hexane, chloroform, ethyl acetate and methanol. It was observed that the EPS dissolved in more polar solvents rather than the non-polar organic solvents indicating the presence of polar substances in the crude EPS. Then, crude EPS isolated from the culture was subjected to thin layer chromatography where various concentrations of mobile phase (10% Methanol: DCM, 5% DCM: Methanol and 10% methanol) were used to move the compound on the plate. Likewise, different detection dyes (p-anisaldehyde, 2, 4-DNP, $KMnO_4$, ninhydrin) were used to detect various compounds which may be present in the crude EPS. The most characteristic spots (three distinct spots) were developed when the compound was moved in 10% DCM: methanol and charred in the solution of potassium permanganate (Figure 5.4).

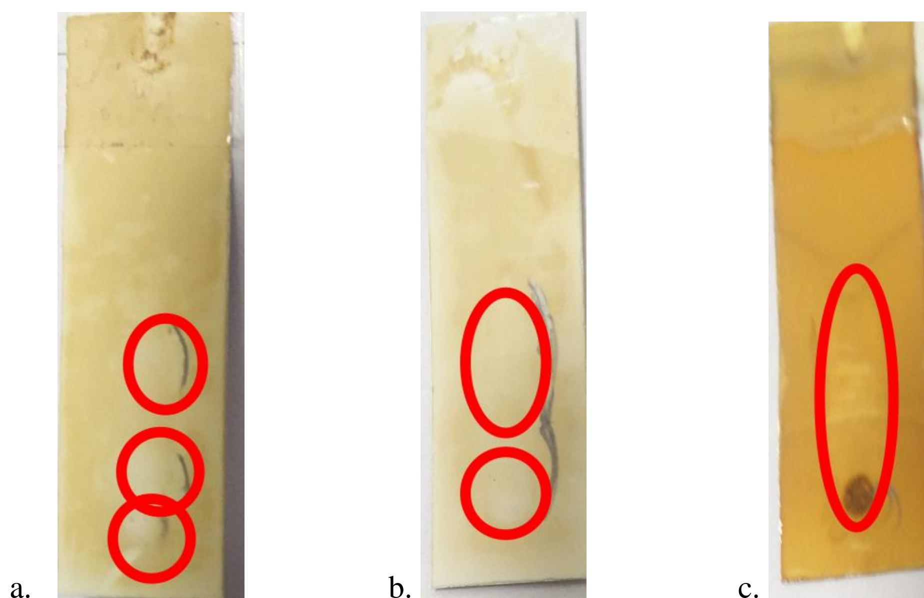

Figure 5.4: TLC analysis of the EPS

*Ethyl acetate (stationary phase) and mobile phase was kept (a) 10% Methanol: DCM; charred in KMnO4, (b) 5% methanol: DCM charred in KMnO4, (c) 10% methanol charred in 2, 4 DNP

5.4. *Fractionation of EPS*

The crude EPS was subjected to silica gel chromatography in order to obtain the fractions. As the polarity of mobile phase was increased, five fractions were eluted. The fractions eluted in solvents were followed by the solvent evaporation by rotary evaporation. First two fractions (DsF1 and DsF2) were obtained when ethyl acetate was used. The fractions were given different name despite of being obtained from same solvent due to the observation of distinct fractions separated by the elution of clear solvent. Even the two fractions were distinguished by colour and smell. Subsequently, the increase of polarity of mobile phase resulted into three different fractions; DsF3 (20% DCM: Methanol), DsF4 (50% DCM: Methanol) and DsF5 (100% methanol). DsF1 had a characteristic fruity smell whereas DsF2 had no smell. DsF3, DsF4 and DsF5 were solid fractions with a bad odour.

5.5. *Estimation of sugar content in the separated fractions*

Phenol sulphuric assessment of all the fractions confirmed the presence of sugar content (Figure 5.5). However, it was found that the polysaccharide content in DsF1, DsF4 and DsF5 was increased by 118, 72 and 42.2% respectively as compared to crude (Table 5.5). DsF1 and DsF4 fractions were focussed for the further study as the sugar content was found to be highest (Figure 5.6).

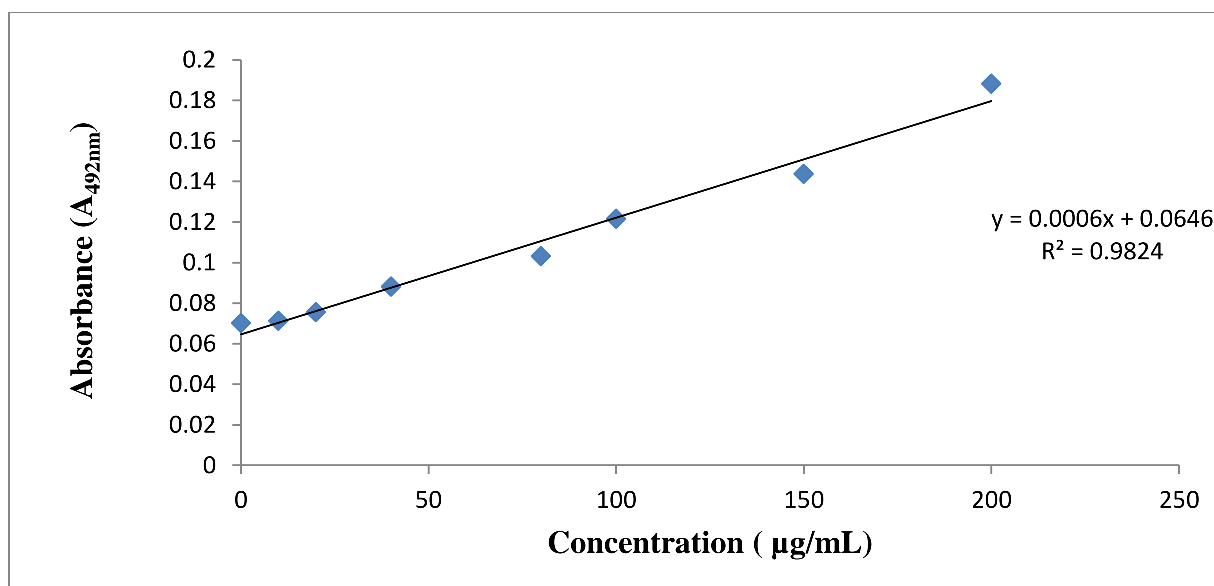

Figure 5.5: Glucose standard curve

Table 5.5: Sugar content in different fractions of EPS

Sample	Glucose equivalents (µg)/ EPS (mg)	Increase (%)
Crude	34.1	_
DsF1	74.4	117.9
DsF2	25.4	-25.6
DsF3	29.2	-14.5
DsF4	58.7	71.9
DsF5	48.5	42.2

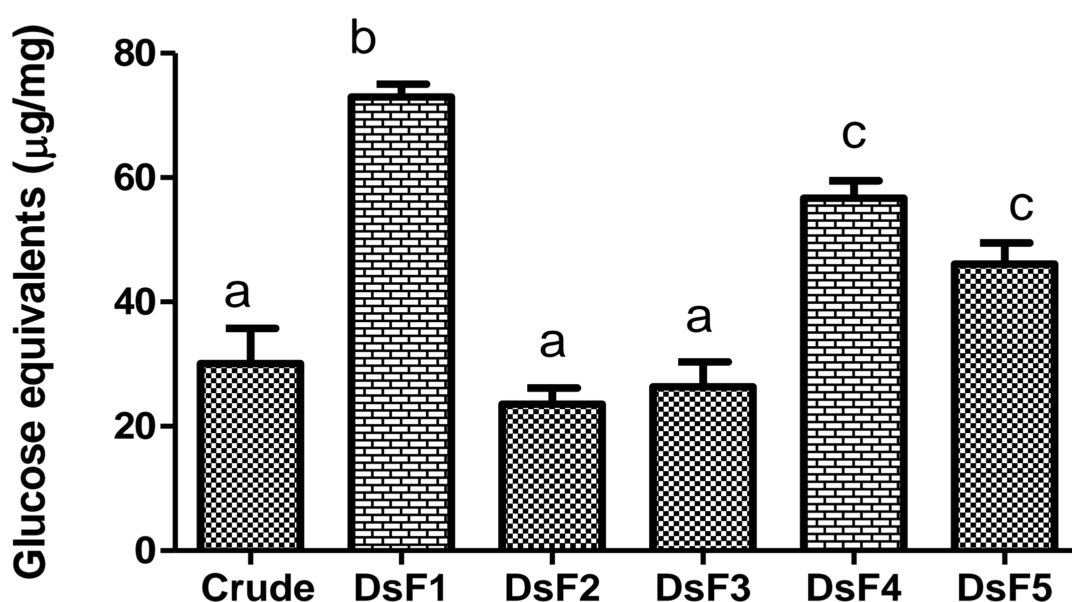

Figure 5.6: Sugar content in different fractions of EPS. Bars with the same lowercase letters are not significantly different at p>0.05.

5.6. Cell growth effect against PBMC

The effect of DsF1 and DsF4 fractions in the concentration of 250, 500 and 1000 µg/mL was assessed on PBMC and compared with crude extract. It was observed that in contrast to the decreasing trend of proliferation index obtained for crude EPS, DsF1 and DsF4 showed the increase in proliferation index with concentration (Figure 5.7 and Table 5.6). In case of DsF1, there is a clear trend of increase in cell viability in concentration dependent manner. The cell viability of DsF1 was observed to be decreased at lower concentration when compared with crude and DsF4 but with increase in concentration, the proliferation index increases. While in

DsF4, although there is no clear trend but the proliferation index is more than 1 indicating the stimulatory effect.

In order to get clear trend, the concentrations were broadened by taking five different concentration (250, 500, 750, 1000 and 1500 µg/mL). Treatment with DsF1 resulted in the dose dependent manner increase in proliferation index and at high concentrations (1000 and 1500 µg/ml), proliferation index was found to be pronouncedly increased. In DsF4, there was no clear trend but at some concentrations, increase in the proliferation index was observed (Figure 5.8 and Table 5.7).

Table 5.6: Comparison of crude, DsF1 and DsF4 EPS on the growth of PBMC

Sample (µg/mL)	Crude	DsF1	DsF4
250	1.05 ± 13.1	0.66 ± 2.3	1.17 ± 29.3
500	0.862 ± 15.6	1.05 ± 13.9	0.98 ± 4.2
1000	0.676 ± 3.3	1.58 ± 9.5	1.19 ± 24.7

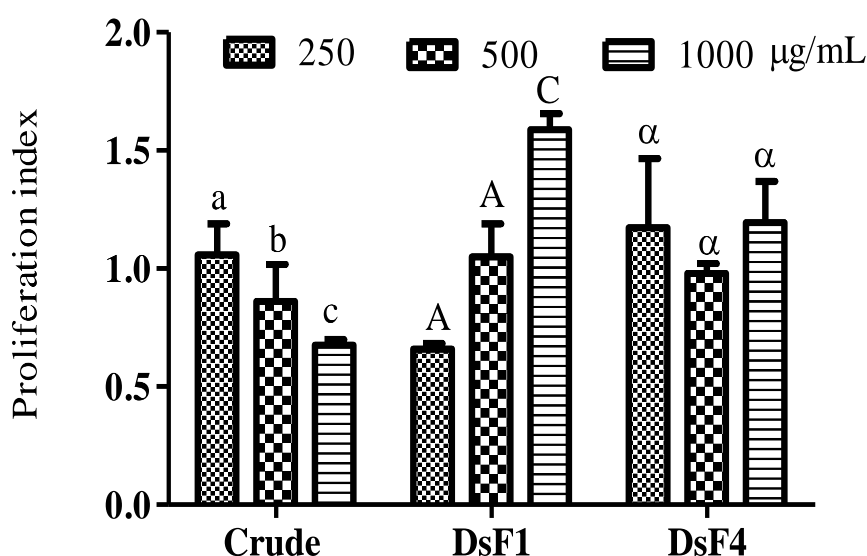

Figure 5.7: Comparison effect of crude, DsF1 and DsF4 EPS on the growth of PBMC. Bars with the same letters are not significantly different at p>0.05. Concanavalin A (5 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the Con A/EPS treated cell and the untreated cells (Cells only).

Table 5.7: Effect of DsF1 and DsF4 on the Proliferation index of PBMC

	Proliferation Index	
Con A*	1.506 ± 23.4	
Sample (µg/mL)	DsF1	DsF4
250	0.549 ± 12.1	1.111 ± 12.8
500	0.757 ± 12.7	1.126 ± 15.9
750	0.897 ± 8.6	1.023 ± 9.3
1000	1.359 ± 12.3	1.101 ± 16.1
1500	1.631 ± 18.1	0.661 ± 3.6

*Con A = Concanavalin A

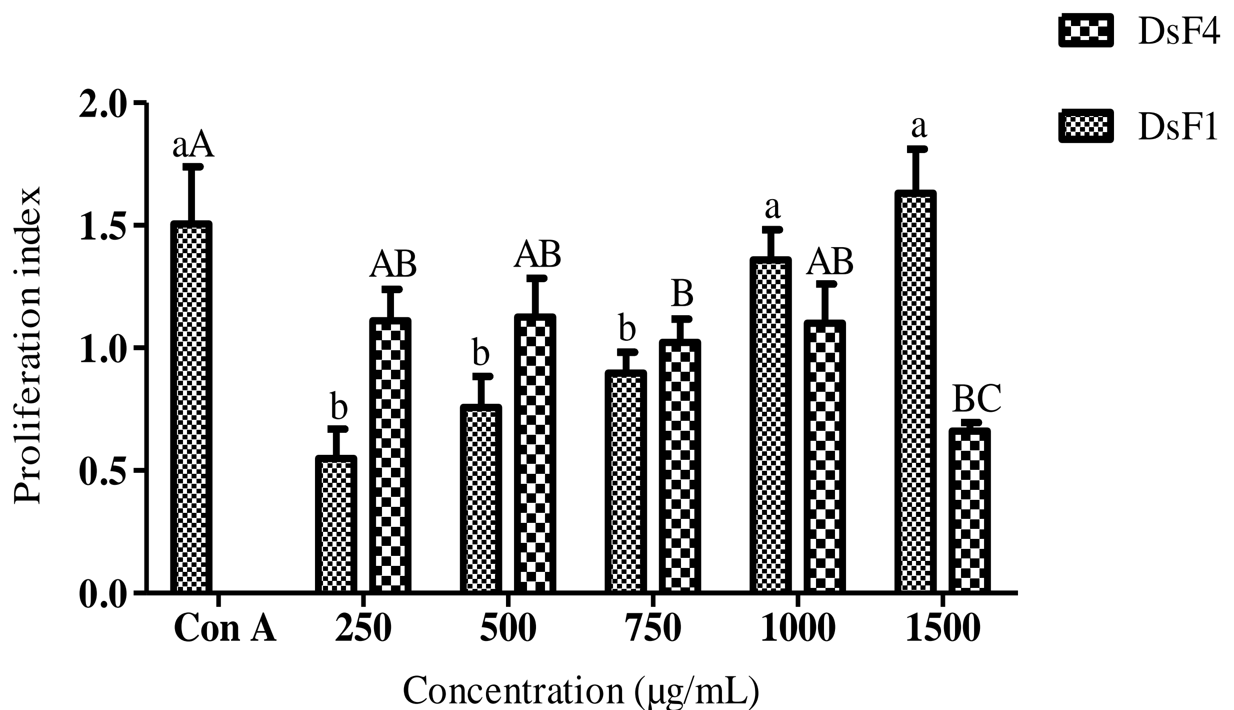

Figure 5.8: Effect of DsF1 and DsF4 on the growth of PBMC. Bars with the same letters are not significantly different at p>0.05. Concanavalin A (5 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the Con A/EPS treated cell and the untreated cells (Cells only).

5.7. Cell growth effect on RAW 264.7 cell lines

The cell growth effect of DsF1 and DsF4 was assessed on RAW 264.7 macrophage cells and the result was compared with crude EPS. Initially, the comparison was made with only three concentrations (250, 500 and 1000 µg/mL). Proliferation index was found to be decreased with increase in concentration in all the cases (Figure 5.9 and Table 5.8). However, most pronounced decreasing effect was observed in DsF1. Proliferation index was found to be more than 1 at lower concentration (250 µg/mL) in each case.

Further, the concentration of DsF1 and DsF4 was broadened (250-1500 µg/ml) to observe the clear trend (Figure 5.10). There was clear trend of decrease in proliferation index observed in DsF1. Apart from 250 µg/mL; all four concentrations had proliferation index less than 1 showing cell growth inhibition effect (Table 5.9). The trend of decreasing proliferation index was also observed in DsF4 but unlike DsF1, cell growth inhibition was only observed in two different concentrations (1000 and 1500 µg/ml) (Figure 5.10).

Table 5.8: Compared growth effect of crude, DsF1 and DsF4 EPS on RAW 264.7 cells

Sample (µg/mL)	Crude	DsF1	DsF4
250	1.23 ± 8.2	1.12 ± 12.6	1.39 ± 15.3
500	0.96 ± 3.3	0.92 ± 9.1	1.02 ± 5.3
1000	0.59 ± 17.3	0.26 ± 0.1	0.82 ± 7.7

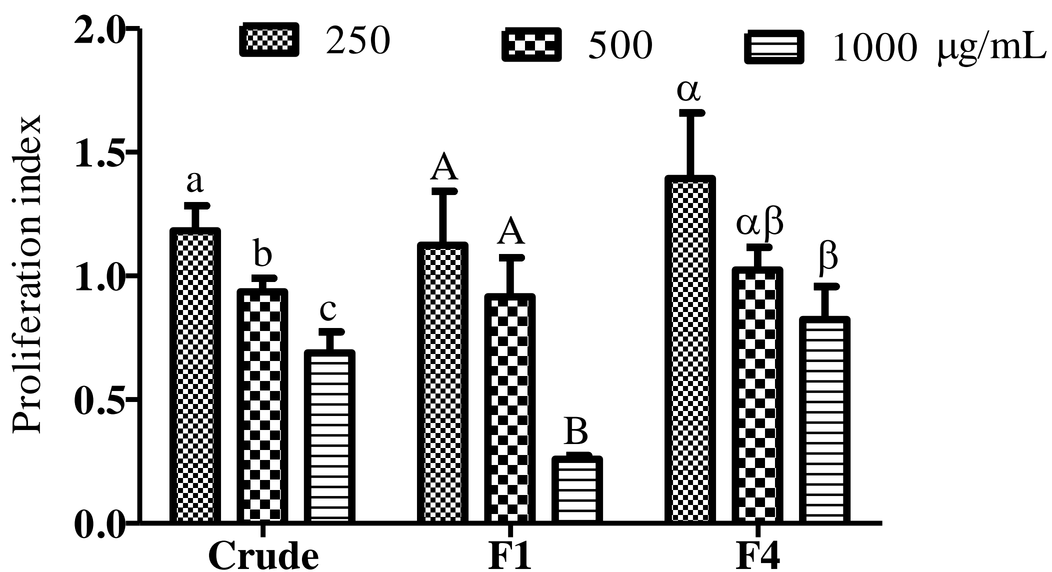

Figure 5.9: Compared growth effect of crude, DsF1 and DsF4 EPS on RAW 264.7. Bars with the same letters are not significantly different at p>0.05. Concanavalin A (5 µg/ml) was used as a positive control. Proliferation index is the ratio of absorbance of the LPS/EPS treated cell and the untreated cells (Cells only).

Table5.9: Effect of DsF1 and DsF4 on the RAW 264.7 cells

	Proliferation Index	
LPS* (2 µg/mL)	1.553 ± 4.5	
Sample (µg/mL)	DsF1	DsF4
250	1.16 ± 12.8	1.361 ± 18.10
500	0.91 ± 9.1	1.02 ± 5.274
750	0.61 ± 1.1	1.27 ± 27.41
1000	0.2590 ± 0.09	0.82 ± 7.742
1500	0.405 ± 0.75	0.44 ± 6.152

*LPS = Lipopolysaccharide

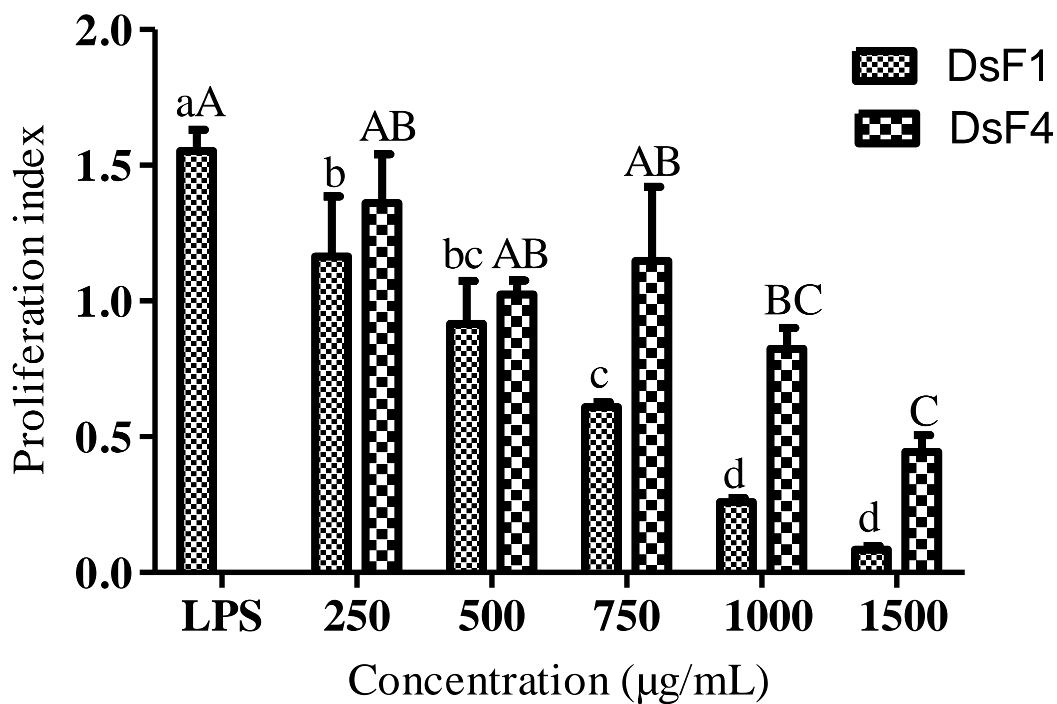

Figure 5.10: Effect of DsF1 and DsF4 on the Proliferation index of RAW 264.7 cells. Bars with the same letters are not significantly different at $p>0.05$. Concanavalin A (5 µg/ml) was used as a positive control. Proliferation index is the ratio of absorbance of the LPS/EPS treated cell and the untreated cells (Cells only).

5.8. Nitric oxide release by RAW 264.7 cell lines

Both the fractions were assessed for the production of nitric oxide from RAW 264.7 macrophage cell lines with the help of griess reagent. NO production was observed to be decreased in DsF1and DsF4 as concentration increased from 250- 1500 µg/mL (Figure 5.11 and Table 5.10). The result is in contrast with that obtained with crude where NO production increased with increasing concentration (Figure 5.3).

Table 5.10: Effect of DsF1 and DsF4 on nitric oxide release from RAW 264.7 cells

	Absorbance	
*Blank	0.09 ± 0.001	
#LPS (2 µg/mL)	0.1 ± 0.0007	
Sample (µg/mL)	DsF1	DsF4
250	0.1 ± 0.0001	0.06 ± 0.006
500	0.07 ± 0.002	0.07 ± 0.002
750	0.05 ± 0.004	0.06 ± 0.0005
1000	0.03 ± 0.003	0.02 ± 0.008
1500	0.02 ± 0.004	0.01 ± 0.003

* Blank = cells only, #LPS = Lipopolysaccharide

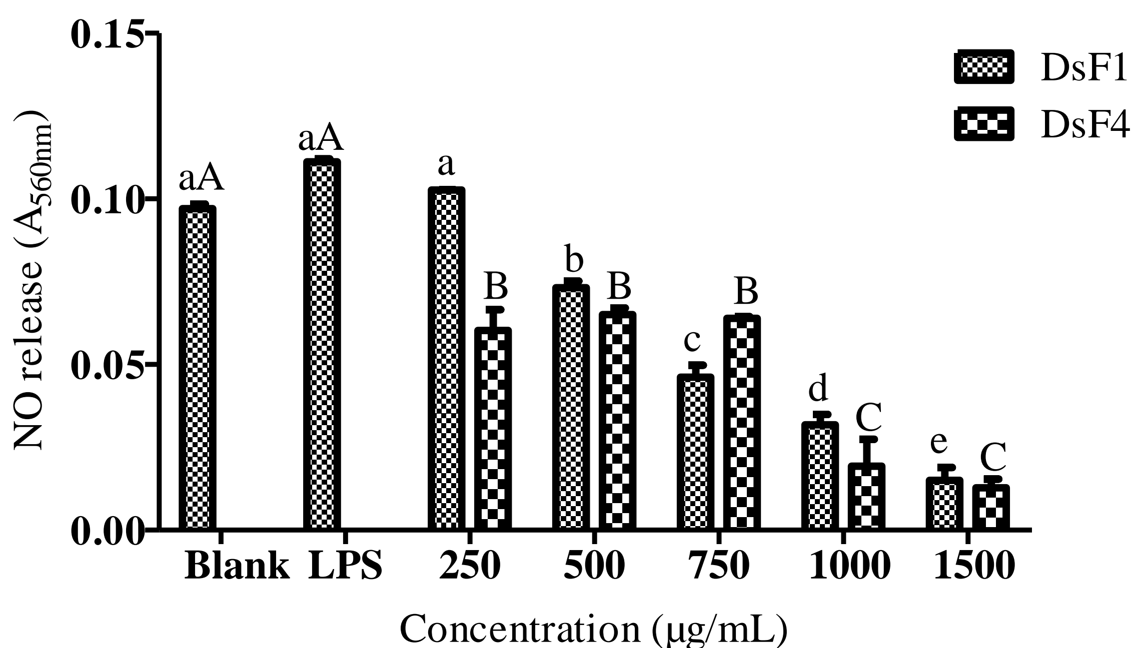

Figure 5.11: Effect of DsF1 and DsF4 on nitric oxide release from RAW 264.7 cells. Bars with the same lowercase letters are not significantly different at $p>0.05$. LPS (2 µg/mL) was used as a positive control.

5.9. Estimation of cytokine Release

In order to assess the production of inflammatory cytokines, three different cytokines (IFN-γ, TGF β and TNF α) levels in PBMC culture for DsF1 and DsF4 were estimated by ELISA.

IFN- γ

DsF4 showed increased cytokine production compared with blank (cells only) but not concentration dependent. Although an increasing trend was observed in DsF1 but the production of cytokine was found to be low up to the concentration of 750 µg/mL compared with blank (Figure 5.12).However, at higher concentration (>1000 µg/mL), the production was increased (Table 5.11, Figure 5.12).

TNF-α

The production of TNF-α was observed to be significantly increasing as the concentration of DsF1 increased (250-1500 µg/mL). In case of DsF4 treatment, the production was found to be increased compared with blank (cells only) but no trend was observed (Figure 5.13, Table 5.12). However, the production of TNF-α was observed to be better in DsF1 as compared to DsF4 (Figure 5.13).

TGF-β estimation

Production of TGF- β was found to be increased with the increase in concentration of DsF1. Like other two cytokines estimated, there was no trend of TGF- β production in DsF4 however at some concentrations, the production was found to more as compared with blank (Figure 5.14, Table 5.13).

Table5.11: IFN γ production in PBMC on treatment with DsF1 and DsF4

	Absorbance	
#Blank	0.13 ± 0.0009	
*ConA	0.17 ±0.002	
Sample (µg/mL)	DsF1	DsF4
250	0.09 ± 0.007	0.17 ± 0.006
500	0.098 ± 0.01	0.13 ± 0.02
750	0.11 ± 0.003	0.14 ± 0.01
1000	0.15 ± 0.003	0.15 ± 0.001

*Con A = Concanavalin A, #Blank = cells only

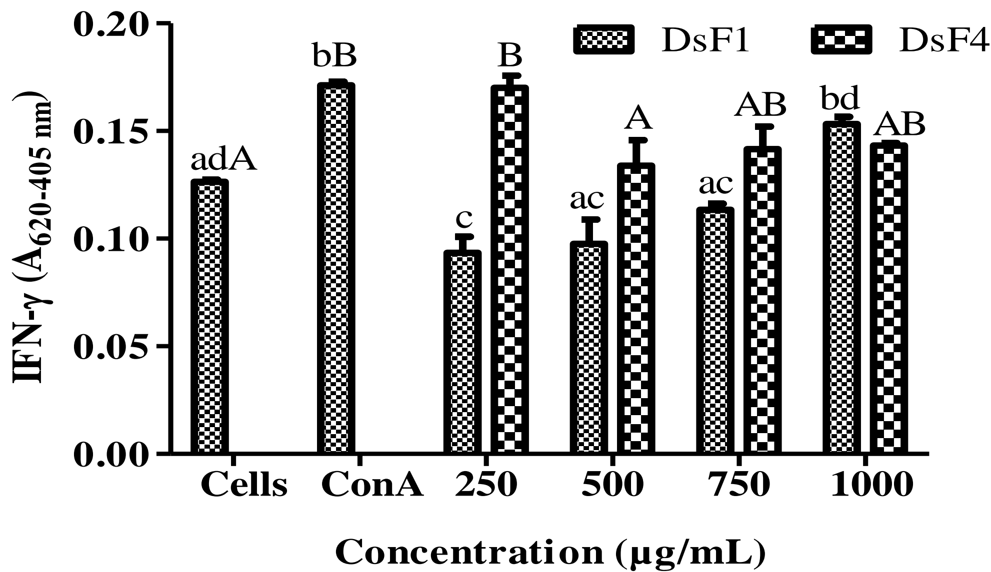

Figure 5.12: IFN γ production in PBMC on treatment with DsF1 and DsF4. Bars with the same letters are not significantly different at p>0.05. Con A: Concanavalin A (5 µg/mL) was used as a positive control.

Table 5.12: TNF- α production in PBMC on treatment with DsF1 and DsF4

	Absorbance	
Blank#	0.14 ± 0.005	
Con A*	0.28 ± 0.003	
Sample (µg/mL)	DsF1	DsF4
250	0.13 ± 0.01	0.11 ± 0.01
500	0.17 ± 0.04	0.14 ± 0.02
750	0.22 ± 0.01	0.16 ± 0.02
1000	0.24 ± 0.07	0.18 ± 0.01
1500	0.25 ± 0.03	0.16 ± 0.02

*Con A = ConcanavalinA, Blank = cells only

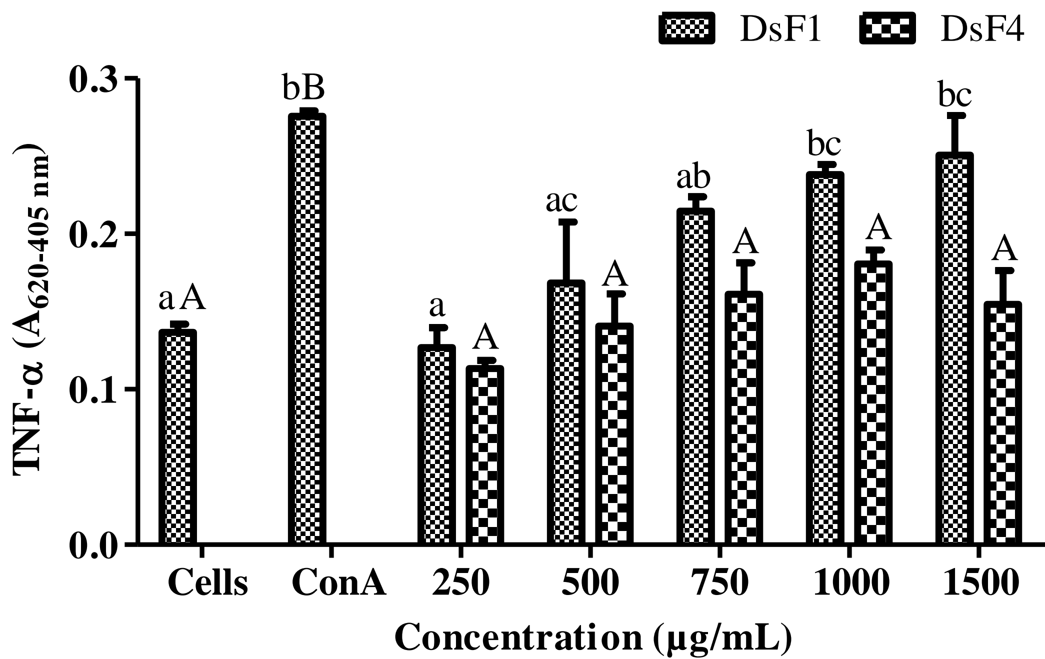

Figure 5.13: TNF-α production in PBMC on treatment with DsF1 and DsF4. Bars with the same letters are not significantly different at p>0.05. Concanavalin A (5 µg/mL) was used as a positive control.

Table 5.13: TGF- β production in PBMC on treatment with DsF1 and DsF4

	Absorbance	
#Blank	0.21 ± 0.002	
*Con A	0.30 ±0.04	
Sample (µg/mL)	DsF1	DsF4
250	0.18 ± 0.002	0.20 ± 0.018
500	0.21 ± 0.001	0.27 ± 0.009
750	0.24 ± 0.003	0.17 ± 0.002
1000	0.26 ± 0.001	0.19 ± 0.024
1500	0.27 ± 0.002	0.22 ± 0.003

* ConA = Concanavalin A, #Blank = cells only

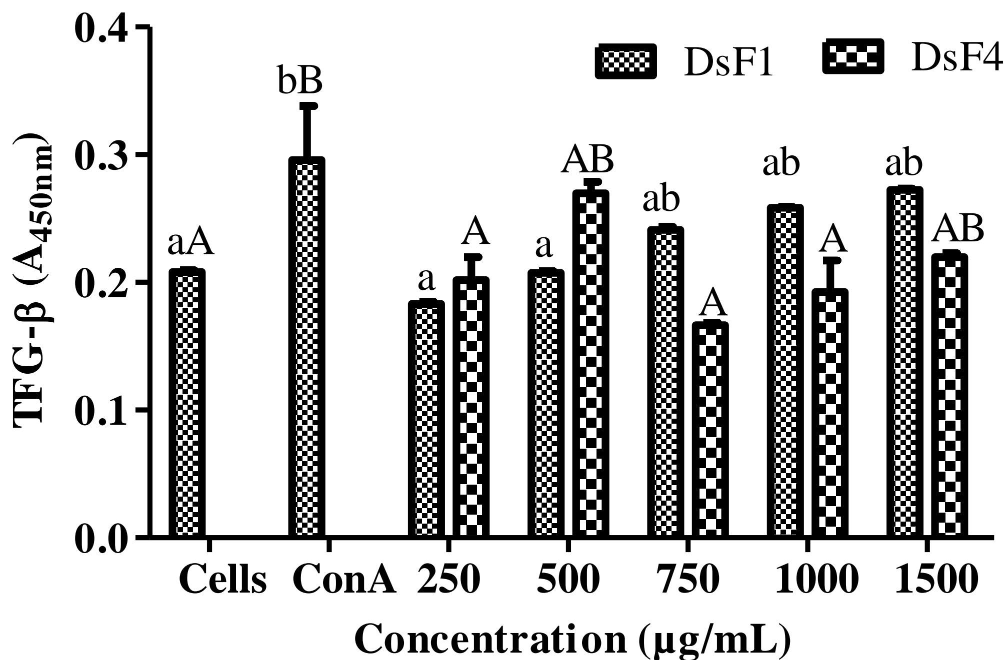

Figure 5.14: TGF- β production in PBMC on treatment with DsF1 and DsF4. Bars with the same letter are not significantly different at p>0.05. Concanavalin A (5 µg/mL) was used as a positive control.

5.10. Anti-oxidant activity assessment

Free radicals are constantly generating in the body which needs to be removed as they lead to various disorders such as carcinogenesis, mutagenesis and aging. Antioxidants are the compounds which are known to remove these free radicals by intervening in the free radical mediated oxidative process.

The anti-oxidant activity of the fractions DsF1 and DsF4 was analysed by DPPH assay. Ascorbic acid was used as positive control. Free radical scavenging activity of both extracts was found to be less (> 20 %) in all the concentration used (250-1500 µg/mL) as compared to crude where activity is increased (Figure 5.15, Table5.14).

Table 5.14: Antioxidant activity of crude, DsF1 and DsF4 EPS

	Antioxidant activity (%)		
*AA	86.2 ± 0.4.		
Sample (µg/mL)	DsF1	DsF4	Crude
250	9.2 ± 0.8	24 ± 2.2	30 ± 0.11
500	12.6 ± 0.2	8.6 ± 1	33 ± 0.17
750	13.7 ± 0.4	8 ± 0.4	50 ± 0.67
1000	14.4 ± 0.9	8.5 ± 0.6	37 ± 1.39
1500	18.1 ± 0.7	9 ± 0.9	43 ± 1.41

*AA=Ascorbic acid

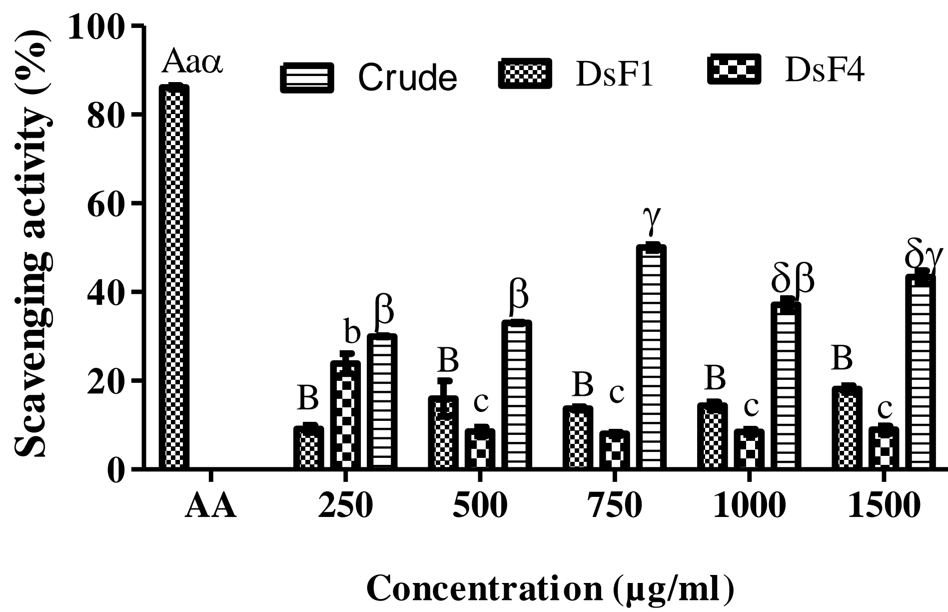

Figure 5.15: Antioxidant activities of crude, DsF1 and DsF4 EPS. Bars with the same letters are not significantly different at p>0.05.*AA = Ascorbic acid

Objective 3: Characterisation of the analysed fractions by Mass spectroscopy and Fourier Transform infrared spectroscopy

5.11. Mass spectroscopy

DsF1 and DsF4 fraction have shown remarkable immune response in both PBMC and RAW264.7 cells. Hence, it was interesting to analyze different bioactive polysaccharides in each fraction. Thus, both fractions were subjected to mass spectroscopy (MS) for the analysis of the compounds present in each fraction. The masses of different polysaccharides present in the fractions were analysed from the spectrum obtained. The higher m/z ratio corresponded to the higher molecular weight polysaccharides. The spectrum obtained showed that DsF4 had comparatively higher molecular weight polysaccharides than DsF1 (Figure 5.16 and 5.17).

The spectra was analysed and the intensities obtained in the spectrum were divided into two divisions (>10000 and >5000) and analysed for the presence of polysaccharides. The mass analysis of the fractions showed the presence of different polysaccharides composed of both hexose and pentose units including tetra-saccharides (4 monosaccharide units), penta-saccharides (5 mono-saccharide units) and even octa-saccharides (eight mono saccharide units) in very high intensities (>10000). DsF1 was found to be comprised of polysaccharides up to five monosaccharide units with association of magnesium and fluorine. However in DsF4 fraction, the higher molecular weight polysaccharides were found with association of different elements such as sulphur, sodium, magnesium, fluorine and chlorine (Table 5.15 and 5.16).

Likewise, the analysis of the m/z ratio lying within the lower intensities (5000-10000) showed close similarities with those found in the intensities >10000 which indicated either the gain or loss of a small ion in order to support the structure of the polysaccharides (Table 5.15 and 5.16). It was also observed that some m/z ratios which were found to be in high intensities in one fraction but had low intensities in another fraction.

Table 5.15: Mass spectra analysis of DsF1 and DsF4 for the intensities observed above 10000

DsF1		
m/z ratio	Intensity	probable compound
181.56	18867	Hexose (Glu/Fruc/Galac/Mann)
224.6	30892	Hexose sugar associated with fluorine
886.54	30368	Penta-saccharide (3 Hexoses+1Pentose + Rhamnose/Fucose)
887.56	18490	Penta-saccharide (3 Hexoses + 1 Pentose +Rhamnose/Fucose)
DsF4		
576.91	11032	Hexose Tri-saccharide (ion associated)
578.9	15626	Hexose Sugar Tri-saccharide (ion associated)
663.63	12803	Tetra-saccharide(2 Hexoses + 2 pentoses)
685.57	35647	Tetra-saccharide
686.59	16242	Tetra-saccharide (Associated with Mg)
1347.23	10190	Octa-saccharide (one group associated with Mg)

Table 5.16: Mass spectra analysis of DsF1 and DsF4 for the intensities observed above 5000

DsF1		
m/z ratio	Intensity	probable compound
207.58	6644	Mg associated hexose
225.62	7349	Hexose sugar associated with fluorine
685.62	6032	Tetra-saccharide
DsF4		
580.89	9896	Hexose sugar tri-saccharide associated with ion
886.53	5929	Penta-saccharide (3 Hexoses + 1pentose + xylose)
1348.23	7710	Octa saccharide (1 group associated with Mg)

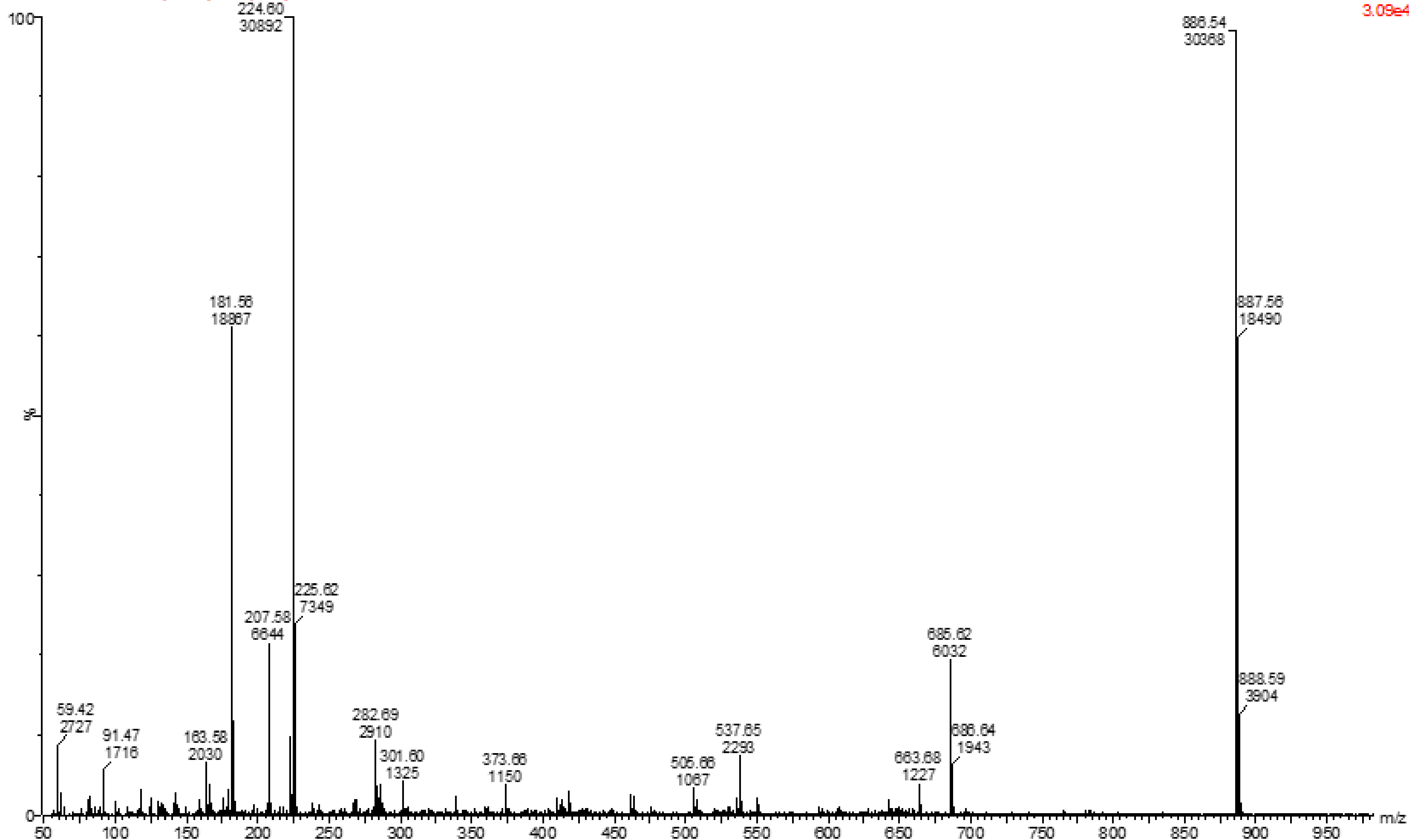

Figure 5.16: Mass Spectrum of DsF1

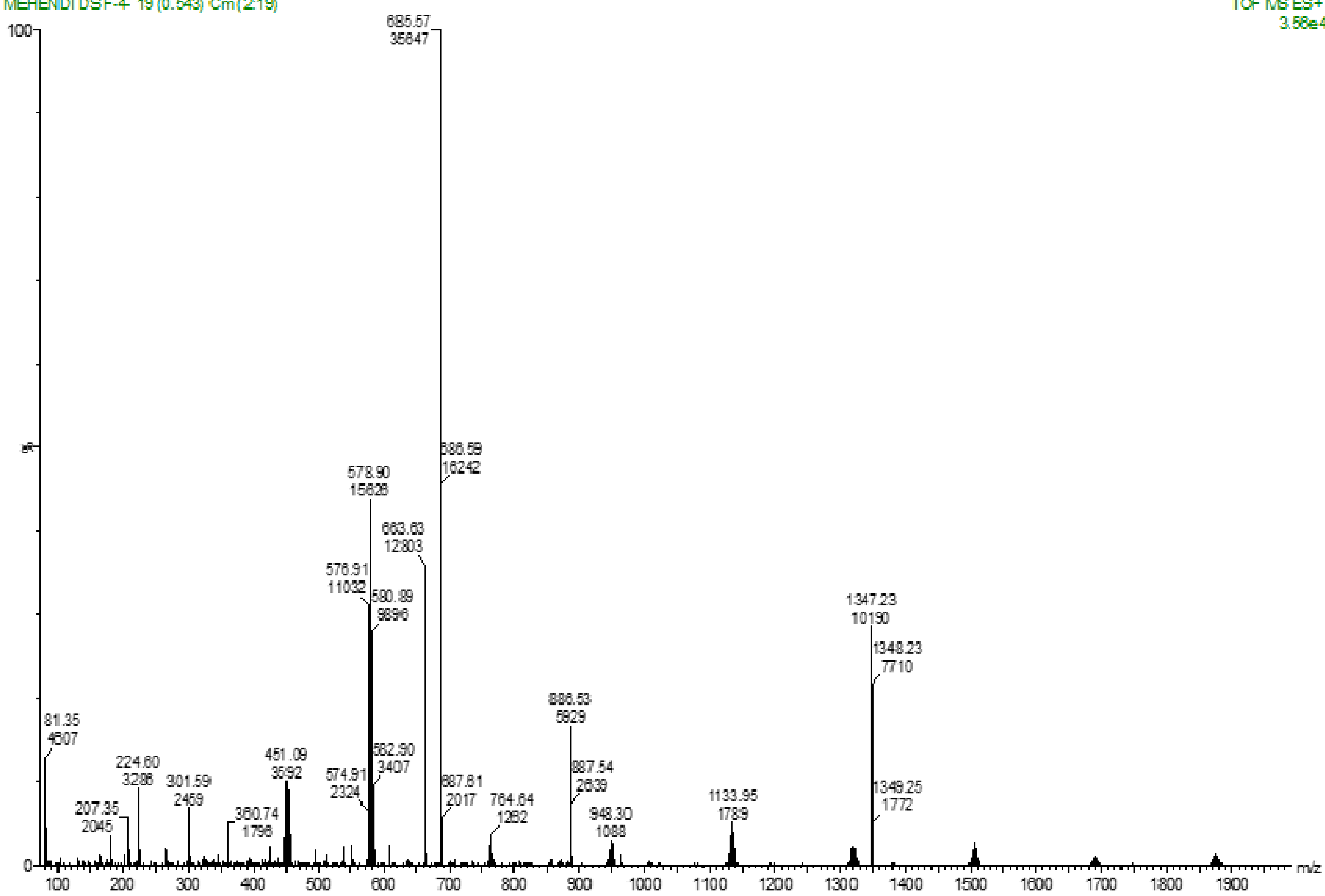

Figure 5.17: Mass Spectrum of DsF4

5.12. *FTIR analysis of DsF1 and DsF4*

FTIR of DsF1 and DsF4 was carried out in order to detect the functional groups present in the sample. The obtained spectra show peaks at certain wavelengths which correspond to the certain chemical group. The depth and intensity of the peak may indirectly correspond to the amount of particular group. FTIR spectra of DsF1 and DsF4 shows the presence of carbonyl (C=O, C-OH) groups in both fractions confirming the presence of polysaccharides (Figure 5.18 and 5.19). Analysis of the spectra of fraction DsF1 obtained revealed that peaks corresponding to the carbonyl group and the halide groups (Table 5.17 and Table 5.18). In contrast, DsF4 showed the presence of the carbonyl groups, halide groups, N-O and –NH groups which may associate to the presence of proteoglycans (Table 5.17 and 5.18).

Table 5.17: FTIR analysis of DsF1

Peak wavelength	Range	Chemical group	Intensity
3387	3200-3600	Alcohol (-OH), (stretch, H-bonded)	Strong. Broad
1614	1620-1610	α,β-unsaturated ketone (C=O)	Strong
1294	1310-1250	aromatic esterC-O (stretch)	Strong
1233	1275-1200	alkyl aryl ether (C-O) (stretch)	Strong
835	850-550	Halo compound (C-Cl), stretching	Strong
786	840-790	Alkene (C=C), bending	Strong, Tri substituted
667	690-515	Halo compound (C-Br), stretching	Strong

Table 5.18: FTIR analysis of DsF4

Peak wavelength	Range	Chemical group	Intensity
3835	3500-3700	Alcohol (-OH), (stretch free)	Strong, Sharp
3741	3500-3700	Alcohol (-OH), (stretch free)	Strong, Sharp
3416	3200-3600	Alcohol (-OH), (stretch, H-bonded)	Strong. Broad
3176	3000-2800	N-H bond, streching	Strong, Broad
2853	3000-2840	Alkane (C-C), stretching	Medium
1678	1678-1668	Alkene (C=C), stretching	Weak, disubstituted
1524	1550-1500	Nitro compound (N-O), stretching	Strong
1327	1310-1250	Aromatic ester (C-O), stretching	Strong
1102	1124-1087	Seconday alcohol (C-O),stretching	Strong
999	995-985	Alkene (C=C), bending	Strong
834	850-550	Halo compound (C-Cl), stretching	Strong
739	730-665	Alkene (C=C), bending	Strong, di substituted
684	690-515	Halo compound (C-Br) stretching	Strong

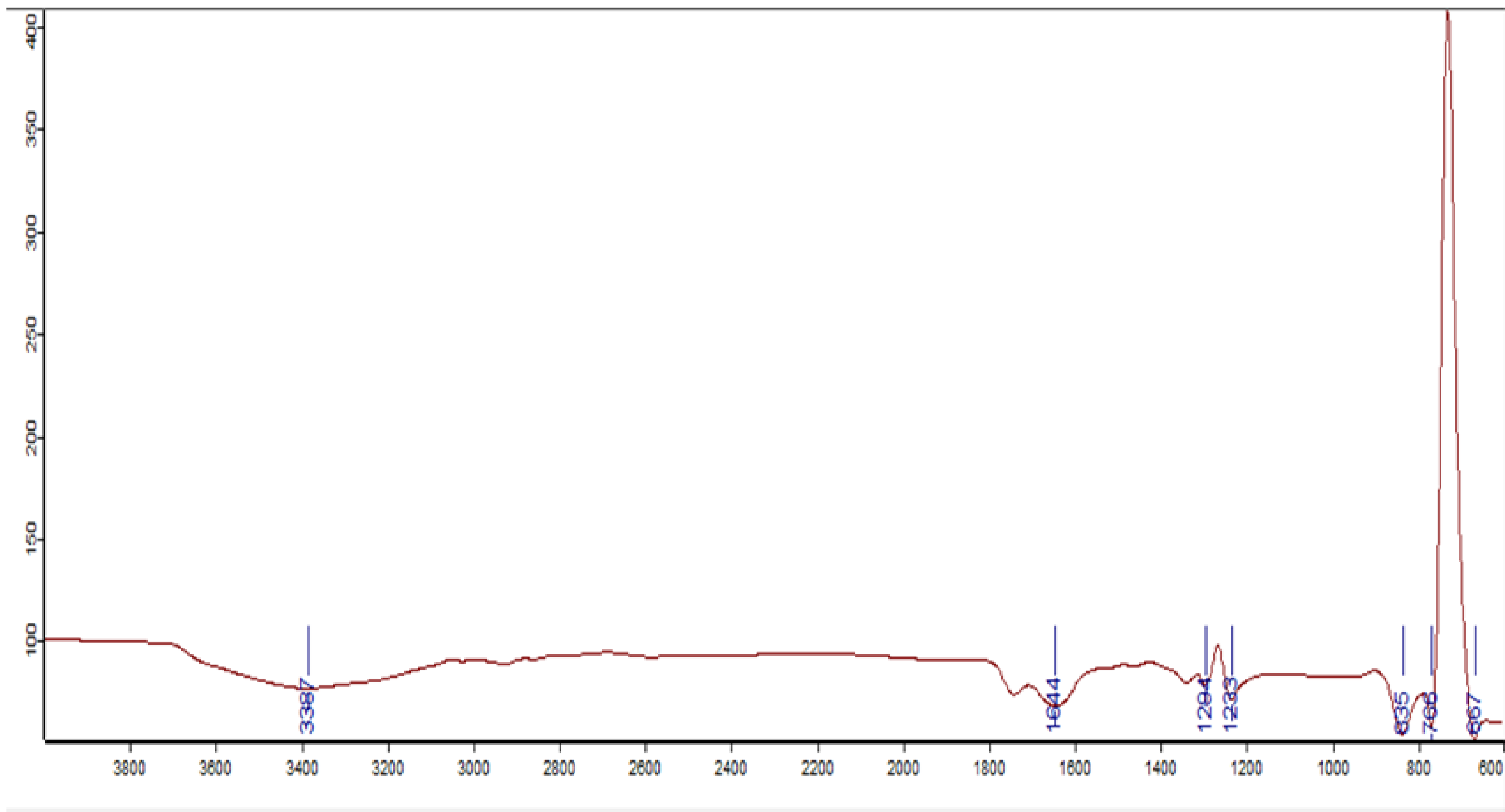

Figure 5.18: FTIR Spectra of DsF1

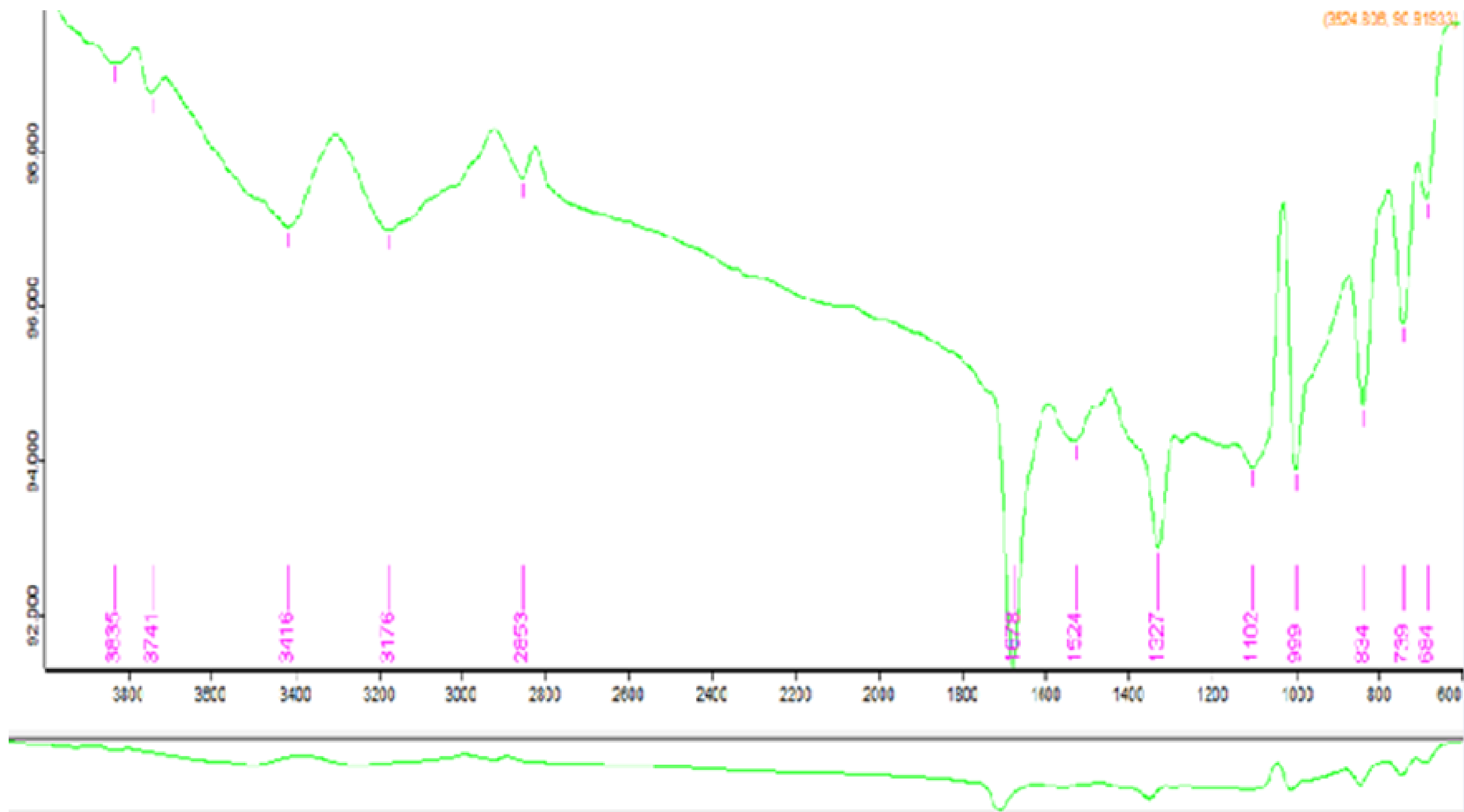

Figure 5.19: FTIR Spectrum of DsF4

Objective 4: Enhancement of sugar content by providing different nutritional and salt stress conditions

5.13. *Growth of D. salina under various stress condition*

After the observation of such remarkable bioactive results from the EPS isolated from the algae grown in the normal condition, next objective of the project was to enhance the production of EPS. To achieve the particular objective, algae was grown in three different stresses (KNO_3: 14.7 mM and 24.5 mM ; KH_2PO_4: 4.2 mM and 7 mM ; NaCl: 3 M and 3.5 M) where normal conditions were 4.9 mM KNO_3 , 1.4 mM KH_2PO_4 and 1.7 M NaCl.

Specific Growth rate

The specific growth rate was calculated by measuring the absorbance at 560 nm in the time interval of 3-4 days. The specific growth rate of the culture grown in stress conditions were compared with normal conditions and it was found to be reduced in all stress conditions (Table 5.19 and Figure 5.20).

Cell count

Along with the specific growth rate, cell count was also measured for 20 days in the same time interval and it was found that biomass (cell number) increased when nitrate and phosphate stress were provided compared to normal condition. However, in case of salt stress, the cell count decreased (Table 5.20). The increase in the biomass was observed when lower concentration of a particular stress was provided (Nitrate: 14.7 mM and Phosphate stress: 4.2mM) but the same was not observed in case of NaCl (Figure 5.21).

Table 5.19: Specific growth rate of *D. salina* cultures in normal and stress conditions

Days	Normal	Stress conditions					
		KNO_3* (14.7mM)	KNO_3(24.5mM)	KH_2PO_4#(4.4mM)	KH_2PO_4(7mM)	NaCl€(3M)	NaCl(3.5M)
3	0.27 ± 0.05	0.037 ± 0.025	0.045 ± 0.004	0.039 ± 0.02	0.108 ± 0.009	0.041 ± 0.02	0.086 ± 0.001
6	0.21 ± 0.09	0.095 ± 0.05	0.169 ± 0.032	0.178 ± 0.02	0.096 ± 0.007	0.104 ± 0.02	0.168 ± 0.03
9	0.098 ± 0.005	0.037± 0.14	0.026 ± 0.012	-0.016 ± 0.02	0.130 ± 0.009	0.066 ± 0.01	0.024 ± 0.01
12	0.099 ± 0.01	0.11 ± 0.14	0.037 ± 0.011	0.023 ± 0.02	0.034 ± 0.01	0.012 ± 0.01	-0.044 ± 0.03
16	0.062 ±0.01	0.021 ± 0.15	0.051 ± 0.07	0.033± 0.007	0.04 ± 0.007	0.09 ± 0.01	0.009 ± 0.01
20	0.061 ± 0.006	-0.02 ± 0.11	0.021 ± 0.07	0.046 ± 0.02	0.052 ± 0.006	0.063 ± 0.006	0.028 ± 0.006

*KNO_3= Nitrate stress, # KH_2PO_4 = Phosphate stress, €NaCl = Sodium salt stress

Table 5.20: Cell counts of the *D. salina* cultures in normal and stress conditions

Days	Normal	Stress conditions					
		KNO_3*(14.7mM)	KNO_3(24.5mM)	KH_2PO_4#(4.4mM)	KH_2PO_4(7mM)	NaCl€(3M)	NaCl(3.5M)
0	0.079 ±0.004	0.011 ± 0.002	0.060 ± 0.05	0.141 ± 0.004	0.09 ± 0.001	0.116 ± 0.006	0.086 ± 0.003
3	0.074 ± 0.001	0.13 ± 0.005	1.49 ± 0.14	0.925 ± 0.009	0.0788 ± 0.008	0.094 ± 0.05	0.08 ± 0.006
6	0.282 ± 0.2	0.178 ± 0.02	0.162 ±0.03	0.055 ± 0.005	0.088 ± 0.001	0.082 ± 0.003	0.104 ± 0.03
9	0.39 ± 0.1	0.58 ± 0.2	0.538± 0.02	0.355 ± 0.09	0.876 ± 0.15	0.798 ± 0.03	0.683 ± 0.06
12	2.962 ±1	6.8 ± 0.2	4.162 ± 0.7	3.825 ± 0.04	3.375 ± 0.15	1.088 ± 1.1	1.275 ± 0.9
16	1.612± 0.1	2.35 ± 0.2	2.15 ± 0.15	1.775 ± 0.14	3.275 ± 0.04	1.088 ± 0.33	1.038 ±1.03
20	1.375 ± 0.1	1.88 ± 0.1	1.9 ± 0.2	1.55 ± 0.1	2.9 ± 0.09	0.864 ± 0.35	0.893 ± 1

*KNO_3= Nitrate stress,#KH_2PO_4 = Phosphate stress, €NaCl = Sodium salt stress

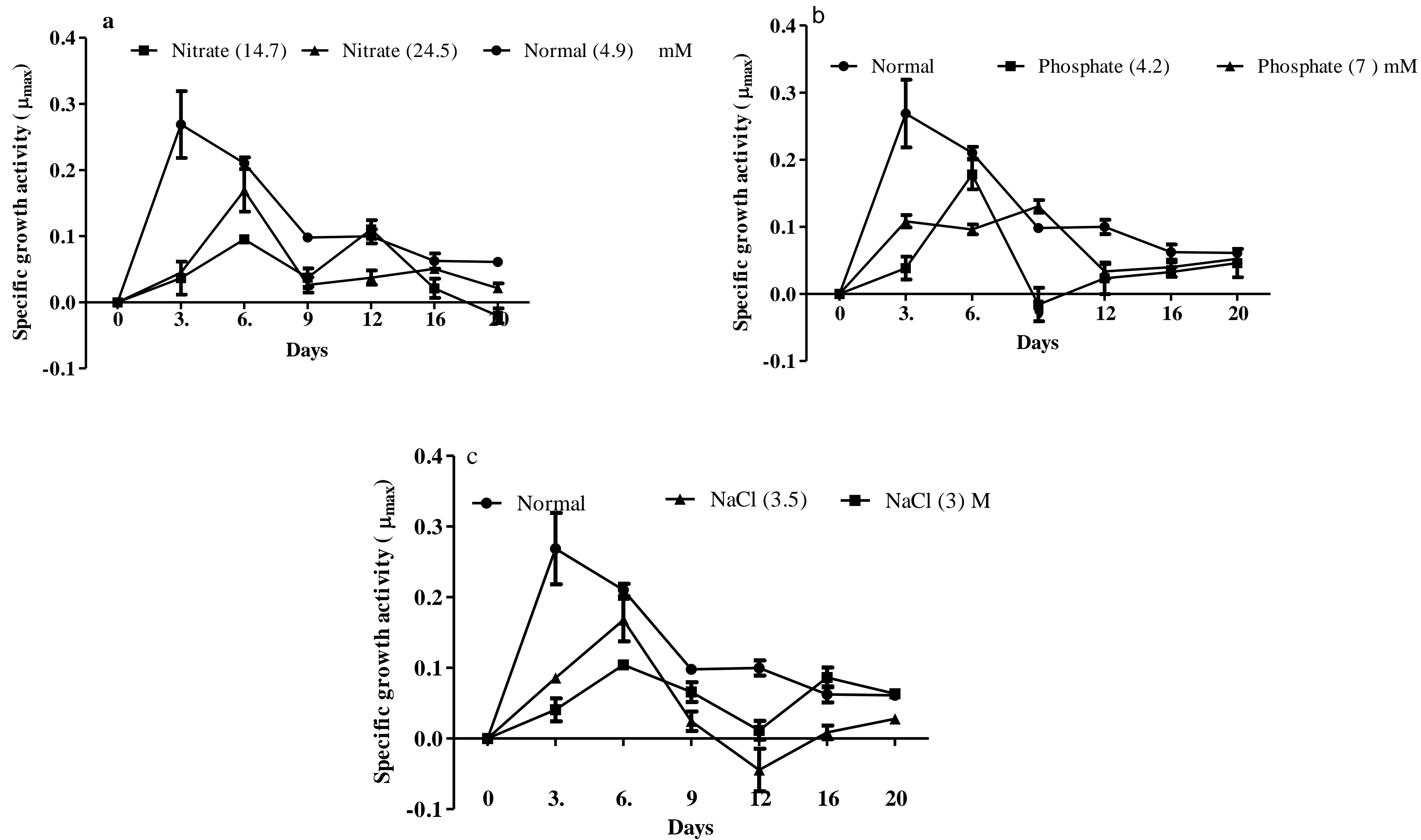

Figure 5.20: Specific growth rate of *D. salina*in different growth stress a) Nitrate b) Phosphate c) NaCl

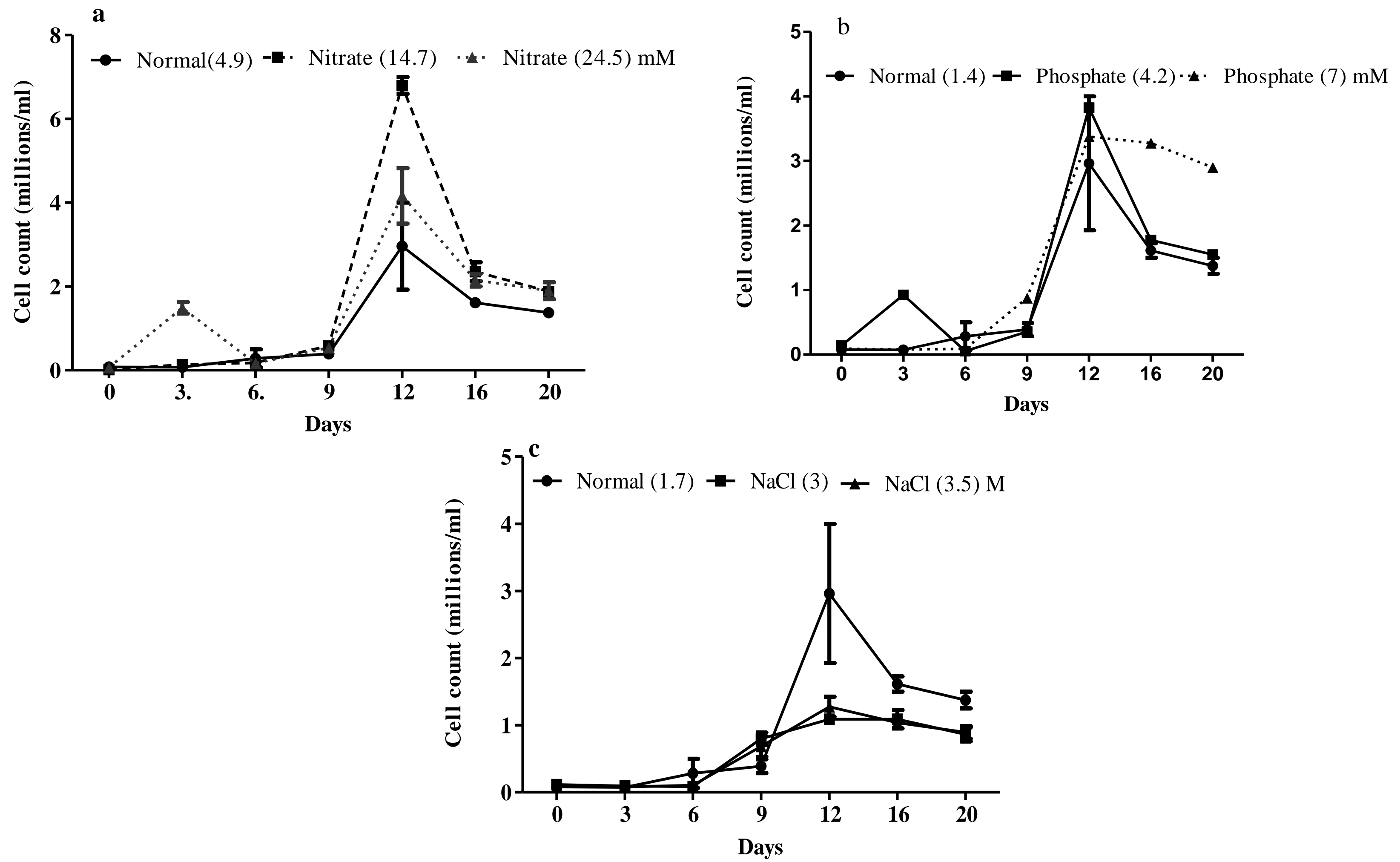

Figure 5.21: Cell count of *D. salina* cells grown in different stress conditions a) Nitrate b) Phosphate c) NaCl

5.14. Estimation of polysaccharides content of EPS obtained from different stress conditions

The EPS were extracted from culture of each stress and proteins were removed by TCA precipitation. The liquid was powdered by lyophilisation and the dried powder was assessed for the presence of sugar. Glucose was kept as standard and the amount of glucose equivalents in EPS were calculated using the standard equation obtained (Figure5.22). It was observed that compared to normal condition, all the stress conditions has caused the increase in sugar content of EPS (Table5.21). However, highest sugar content was observed in salt stress (NaCl, 3 M) (Figure5.23).

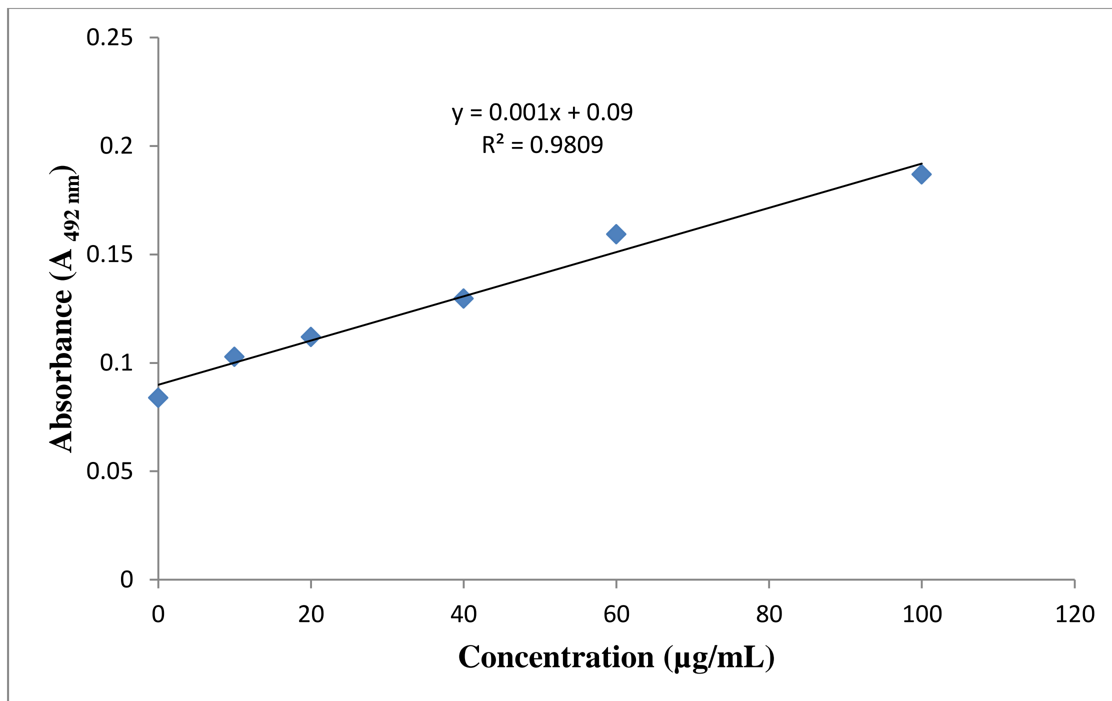

Figure 5.22: Standard curve of glucose

Table5.21: Sugar content in EPS secreted from different stress and normal conditions

Stress	Glucose equivalents(µg) / EPS (mg)	Fold increase*
Normal	7.7 ± 0.3	
KNO_3 (14.7 mM)	22.3 ± 2.4	2.9
KNO_3 (24.5 mM)	19.6 ± 1.7	2.5
KH_2PO_4 (4.4 mM)	20.1 ± 1.8	2.6
KH_2PO_4 (7 mM)	15.7 ± 2	1.2
NaCl (3 M)	88 ± 2.5	11.4
NaCl (3.5 M)	29.5 ± 0.6	2.5

*Fold increase: Stress/Normal

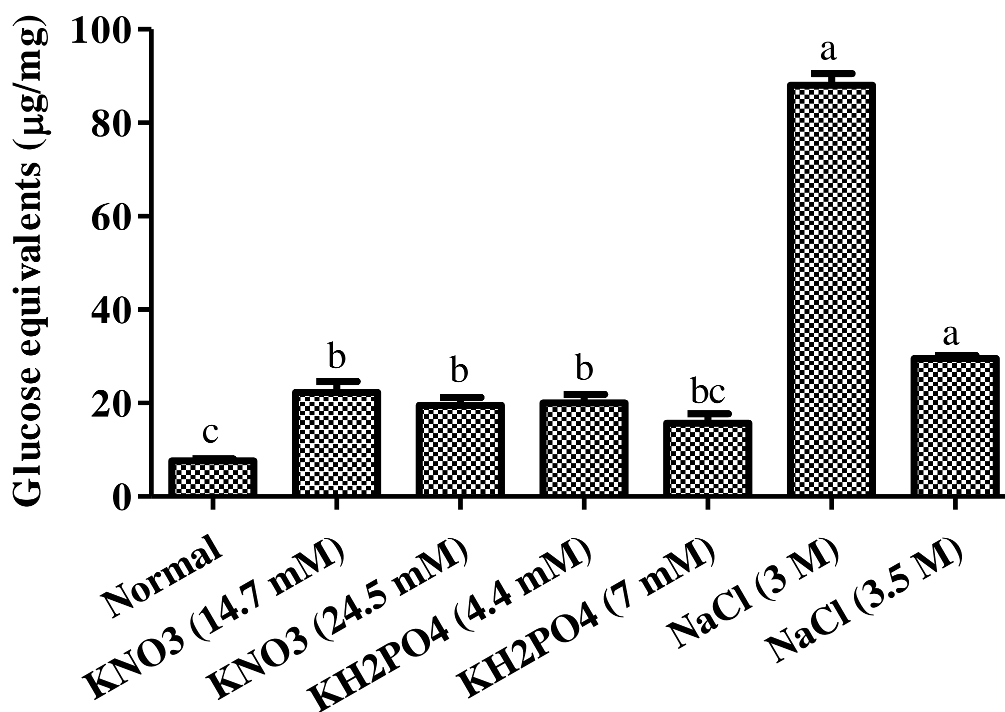

Figure 5.23: Sugar content in EPS secreted from different stress provided and normal conditions. Bars with the same letters are not significantly different at $p>0.05$.

5.15. Anti-oxidant activity of EPS isolated from stress conditions

Antioxidant activity of the EPS obtained from different stress conditions was measured as means of scavenging DPPH. The antioxidant activity of the EPS obtained from all the stress conditions was more than EPS of normal conditions at lower concentration (Table 5.22). Within a particular stress condition, the anti-oxidant activity was found to be increased as the concentration increased (Table 5.22). However, enhanced activity was shown by EPS obtained from culture growing in NaCl stress (3M, 3.5 M) as the concentration increased (Figure 5.24)

Table 5.22: Free radical scavenging activity of the EPS isolated from different stress and normal conditions.

	Free radical scavenging activity (%)	
AA*	74.43	
Stress	250 µg/mL	1000 µg/mL
Normal	29.88	38.5
KNO_3 (14.7 mM)	35.13	30.23
KNO_3 (24.5 mM)	35.52	37.38
KH_2PO_4 (4.4 mM)	35.68	29.38
KH_2PO_4 (7 mM)	31.75	36.37
NaCl (3 M)	36.12	46.27
NaCl (3.5 M)	31.42	51.26

*AA = Ascorbic acid

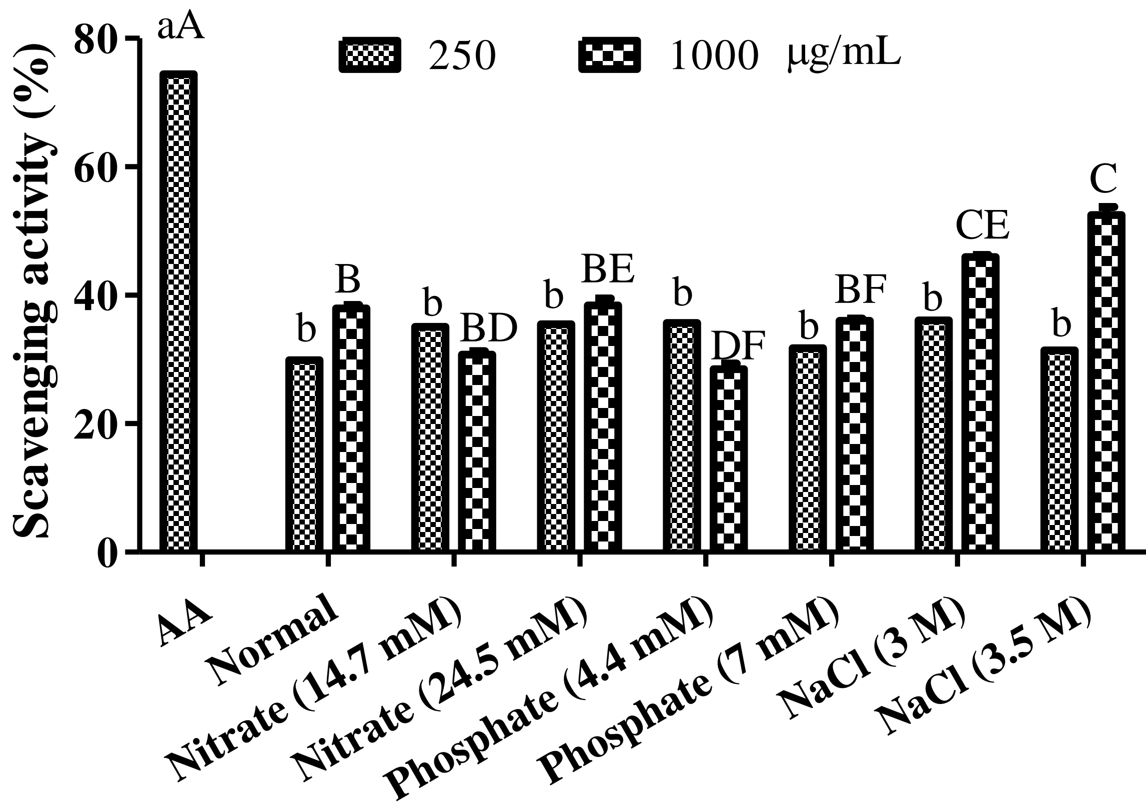

Figure 5.24: Free radical scavenging activity of the EPS isolated from different stress and normal conditions. Bars with the same letters are not significantly different at $p>0.05$. AA = Ascorbic acid

Chapter VI

Discussions

6. Discussions

Extracellular polysaccharides from micro origin have gained recent attention in various biotechnological and biomedical applications. Several microalgae such as *Chlorella, Ascophyllum, Palmeria, Dunaliella* and *Porphyra* are reported to produce polysaccharides extracellularly (de Jesus Raposo *et al.* 2015a). These polysaccharides are known to have antioxidant, anti-tumour, anti-glycemic, anti-lipidimic, anti-viral, anti-bacterial and immunomodulatory activities (de Jesus Raposo *et al.* 2015b). *D. salina* is reported to produce polysaccharide extracellularly but their applications are mainly focused in food industry (Ben-Amotz & Avron 1989). Studies on the exploration of EPS isolated from *D. salina* showing bioactive properties are limited. In present study, two fractions (DsF1 and DsF4) of EPS obtained by chromatography having high sugar content exhibited immunomodulatory effect against both PBMC and RAW264.7 cells. Mass spectra analysis confirmed the presence of the polysaccharide composed of both hexose and pentose sugars and FTIR results showed the presence of functional groups associated with the polysaccharides. Polysaccharide content was found to be increased when the algae was grown under stress conditions.

Immunomodulators are the response modifier bio-compounds which are known to be present either as a stimulator or suppressor of immune system and have been widely used for several therapeutic purposes. EPS isolated from *Porphyridium tricornutum* and *Chlorella stigmatophora* have shown immunostimulatory and immunosuppressant activity against PBMC and macrophages respectively (Guzman *et al.* 2003). Polysaccharides isolated from *Ulva rigida* activate the production of nitric oxide and cytokines from RAW 264.7 macrophage cells (Ray & Lahaye 1995). In agreement with these findings, crude polysaccharides from *D. salina* have shown increase in cell proliferation at lower concentration whereas growth inhibition at higher concentration on both PBMC and RAW 264.7 macrophages. Nitric oxide estimated in RAW 264.7 cells has shown a trend of increase in NO production with concentration of crude EPS. Variable immunological effect at different concentrations may be due to the mixture of compounds in crude EPS. Thus TLC was carried out showing three tailed spots suggesting the presence of different compounds. Previously, the size exclusion chromatography has been used to separate the fraction of polysaccharides isolated from *Taxillus chinensis* and *Uncaria rhyncophylla* to obtain threes and two fractions respectively reporting the enhancement of NO and TNF-α production in J774 A.1 mouse macrophages (Zhang *et al.* 2013). In one study, EPS from D. salina was

fractionated using DE-52 ion exchange chromatography to obtain two fractions (Xu *et al.* 2007). Here, the crude EPS was fractionated by silica gel chromatography to obtain five different fractions. The fractions thus obtained were analysed for the sugar content and two fractions (DsF1 and DsF4) containing high amount of sugar were focussed for the assessment of bioactivity. In PBMC, there was increase in cell proliferation with increase in concentration of both fractions. Cytokines plays key role in regulating immune system hence, the production of three cytokines (IFN-γ, TGF-β and TNF-α) was estimated in the PBMC culture. The concentration dependent increase in the production of these cytokines was observed in both DsF1 and DsF4. Both fractions exhibited increased cell proliferation and cytokines production in PBMC which is indicator of immunoProliferation. While in case of RAW cells, both fractions were found to exhibit dose dependent inhibitory effect on cell growth and nitric oxide production. As reported in previous studies (Singh *et al.* 2016), Crude and fractions were found to be positive for free radical scavenging activity although the activity was less in the fractions.

Infrared spectroscopy reveals the presence of functional groups present in the compound as the molecules at specific frequencies can rotate or vibrate corresponding to the discrete energy levels (Nicolet & All 2001). Single functional group is usually associated with adjacent group which leads to the variation in frequency in stretched or bending modes of vibration. The FT-IR spectra are interpreted by correlating the absorption spectra of unknown compound with the known absorption peaks for different functional groups. Previously, FT-IR spectroscopy in *D. salina* has been performed for the crude EPS isolated from *D. salina* and observed peaks of –NH, C-O, C-X, C-N, N=C=O and C-C groups (Mishra & Jha 2009). In contrast to this study where crude EPS was analyzed, current study deal with fractionated EPS. DsF1 confirmed the presence of –OH, C=O, C-O, C-X and C=C groups where as DsF4 showed the presence of C-C, C=C, C=O, C-O, N-H, N=O and C-X functional groups.

MALDI-TOF analysis of crude EPS in *D. salina* represented the m/z ratio corresponding to the deprotonated hexose sugar, disaccharide (hexoses and pentoses), Mg associated disaccharide (hexoses and pentoses) and tetrascahharides in previous study (Mishra *et al.* 2011). This study revealed the presence of three different kinds of sugar moieties in the EPS i.e. aldohexoses (glucose and galactose), ketokexoses (fructose) and pentoses (xylose). The presence of four monosaccharides (glucose, galactose, fructose and xylose) has already been confirmed in previous studies through HPLC that too in the crude EPS (Mishra & Jha 2009). However, the present study carried the mass analysis of fractionated EPS (DsF1 and

DsF4).The obtained m/z ratios revealed that in DsF1 and DsF4, all the reported moieties were found. Apart from that, m/z corresponding to the polysaccharides with up to eight monosaccharide units (m/z = 1347.23) was also found to be present in DsF4 whereas in DsF1, the highest m/z ratio (887.56 and 886.54) corresponded to the presence of polysaccharides with up to five monosaccharide units.

D. salina is known to sustain itself in wide range of stresses (Borowitzka & Borowitzka 1988). It is also known that the production of metabolites increases when the alga is put under stress condition as observed in the production of β-carotene and glycerol (Ben-Amotz 1980, 1995). Likewise the production of EPS is also greatly influenced by the algal growth in an environment where the cells are in stress conditions. In a study, the EPS production was found to be increased in salt stress (Mishra & Jha 2009). In accordance with this, our study also showed increased EPS production in salt stress. Present study has also looked for nutrient stress (nitrate and phosphate) and it was found that the EPS production enhanced as compared to normal conditions.

Chapter VII

Conclusions

7. Conclusion

Metabolites from *D. salina* such as β-carotene and glycerol have already been commercialised and associated with several therapeutic purposes. Dried whole organism is also marketed as the complete source of proteins, carbohydrates, lipids, carotenoids and energy. In the present study, two high sugar containing fractions (DsF1 and DsF4) of EPS have shown immunostimulatory effect by enhancing the cell growth and cytokines (IFN-γ, TGF-β and TNF-α) production in PBMC. Inhibitory effect was observed in RAW 264.7 cells as these two fractions diminished the cell growth and NO production. Further, the characterisation of the fractions by mass spectroscopy revealed the presence of hexoses and pentoses in the fractions in variable amounts. The FTIR data confirmed the presence of halide associated sugars and even proteoglycans. The study also reported the enhancement of EPS production by growing the culture in stress conditions. Hence, these polysaccharides may be exploited commercially as immunomodulators in therapeutics.

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