

**THE EFFECTS OF *Nannochloropsis* sp.
METHANOLIC EXTRACT ON PREVENTION OF
DNA DAMAGE IN L929 CELLS**

HAZIQ AHMAD HAZWAN BIN ZAINODDIN

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by

HAZIQ AHMAD HAZWAN BIN ZAINODDIN

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LIST OF SYMBOLS

μL	microlitre
mg	milligram
μg	microgram
ml	millilitre
g	gram
m	meter
mm	millimetre
μM	micrometre
$\mu\text{g/ml}$	microgram per millilitre
mg/ml	milligram per millilitre
ml/min	millilitre per minute
$\mu\text{L/min}$	microlitre per minute
IC_{50}	50% Inhibitory Concentration
$^{\circ}\text{C}$	degree celcius
%	percentage

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
DR	Direct Repair
BER	Base Excision Repair
NER	Nucleotide Excision Repair
MMR	Mismatch Repair
DSBR	Double Strand Break Repair
DPPH	1,1- Diphenyl-2- Picryl- Hydrazyl
H ₂ O ₂	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
IC ₅₀	50% Inhibitory Concentration
L929	Mouse Fibroblast Adipose Tissue
mM	milliMolar
mg	milligram
TE	Tris-EDTA

**KESAN EKSTRAK METANOLIK *Nannochloropsis* sp. TERHADAP
PENCEGAHAN KEROSAKAN DNA DALAM SEL L929**

ABSTRAK

Nannochloropsis dan *Spirulina* adalah beberapa spesies mikroalga yang didapati mempunyai pelbagai aktiviti biologi seperti antioksidan dan sifat antikerosakan DNA. Malangnya, masih kurang kajian tentang kesan kedua-dua mikroalga terutamanya spesies *Nannochloropsis*. Oleh itu, kajian ini dijalankan untuk menentukan kesan *Nannochloropsis* sp. ekstrak pada pencegahan kerosakan DNA dalam lapisan sel L929. Fitokimia kedua-dua mikroalga telah diekstrak menggunakan 100% metanol. Pengekstrakan kemudiannya disaring dengan HPLC untuk mengesan kehadiran komponen yang disasarkan. Analisis HPLC menunjukkan bahawa *Nannochloropsis* sp. ekstrak metanol mempunyai asid kafeik, asid *p*-coumaric, naringenin dan hesperetin manakala *Spirulina* sp. ekstrak methanol hanya mempunyai asid kafeik, quercetin dan naringenin. Ekstrak kemudiannya diuji untuk Jumlah Kandungan Fenolik (TPC) dan Jumlah Kandungan Flavonoid (TFC). Keputusan menunjukkan bahawa ekstrak untuk kedua-dua TPC dan TFC untuk *Nannochloropsis* sp. lebih tinggi daripada *Spirulina* sp. dengan 58.43 mg GAE/g dan 19.64 mg GAE/g, dan 79.87 mg QE/g dan 11.19 mg QE/g masing-masing. Kajian kemudiannya diteruskan dengan menilai aktiviti antioksidan bagi ekstrak mikroalga menggunakan analisis DPPH-HPLC. *Nannochloropsis* sp. ekstrak metanol mempamerkan peratusan perencatan DPPH yang lebih tinggi berbanding *Spirulina* sp. ekstrak. Korelasi negatif wujud antara TPC dan TFC dengan EC₅₀ aktiviti perencatan antioksidan DPPH. Dalam kajian ini, *Spirulina* sp. dan *Nannochloropsis* sp. menunjukkan ketoksikan sederhana terhadap lapisan sel L929 dengan IC₅₀ masing-masing 1.701 mg/mL dan

1.134 mg/mL. Dalam analisis kerusakan DNA dengan *Nannochloropsis* sp., keputusan menunjukkan bahwa ekstrak metanol mampu mencegah dan mengurangi aktivitas kerusakan DNA. Oleh itu, mikroalga boleh dicadangkan sebagai salah satu bentuk alternatif bahan ubatan yang berpotensi dalam industri perubatan dan farmaseutikal.

**THE EFFECTS OF *Nannochloropsis* sp. METHANOLIC EXTRACT ON
PREVENTION OF DNA DAMAGE IN L929 CELLS**

ABSTRACT

Nannochloropsis and *Spirulina* are some of microalgae species that have been found to have a wide range of biological activities such as antioxidant and anti-DNA damage properties. Unfortunately, there is still lack of study on the effect of both microalgae especially *Nannochloropsis* species. Hence, this study was carried out to determine the effect of *Nannochloropsis* sp. extract on prevention of DNA damage in L929 cell line. The phytochemicals of both microalgae were extracted using 100% methanol. The extractions were then screened with HPLC to detect the presence of targeted compounds. HPLC analysis revealed that *Nannochloropsis* sp. methanolic extracts had caffeic acid, *p*-Coumaric acid, naringenin and hesperetin while *Spirulina* sp. methanolic extract had caffeic acid, quercetin and naringenin. The extracts were then tested for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). Results revealed that the extracts for both TPC and TFC for *Nannochloropsis* sp. were higher than *Spirulina* sp. with 58.43 mg GAE/g and 19.64 mg GAE/g, and 79.87 mg QE/g and 11.19 mg QE/g respectively. The study was then proceeded by evaluating the antioxidant activities of the microalgae extracts using DPPH-HPLC analysis. *Nannochloropsis* sp. methanolic extract exhibit greater percentage of DPPH inhibition compared to *Spirulina* sp. extract. A negative correlation existed between TPC and TFC with the EC₅₀ of DPPH antioxidant inhibition activity. In this study, *Spirulina* sp. and *Nannochloropsis* sp. showed moderate toxicity toward L929 cell lines with IC₅₀ 1.701 mg/mL and 1.134 mg/mL respectively. In DNA damage analysis with *Nannochloropsis* sp., the result revealed that the methanolic extract are able to prevent

and reduce DNA damage activity. Thus, microalgae can be proposed as one of alternative form in medical and pharmaceutical industry.

CHAPTER 1

INTRODUCTION

1.1 Research backgrounds

Microalgae are species that belong to Kingdom Plantae based on taxonomy classification. These microalgae, cyanobacteria, and diatoms are diverse group of unicellular organisms and can be found in different shapes and forms: spherical or filamentous, elongated and their cell size ranges from 0.5 μm to 200 μm (Roy and Mohanty, 2019) but barely can be seen without microscope. Microalgae have the capability to undergo photosynthesis due to the chlorophyll content in their cells (Benavente-Valdés *et al.*, 2016). The capability of performing photosynthesis is crucial for the earth as the total oxygen produced from photosynthesis approximately half of the atmospheric oxygen while constantly take-out carbon dioxide in atmosphere for photoautotrophic growth (Mondal *et al.*, 2017).

In general, microalgae can be divided into two types; fresh water and marine microalgae (Chu *et al.*, 2002). They can be found in both terrestrial and aquatic ecosystems. Approximately, about 168,999 species and infraspecific names, 23,021 images, 65,784 bibliographic items and 520,816 distributional records processed by AlgaeBase till December 2022 (AlgaeBase, 2023). The first cultivation of microalgae was done in Japan back in 1960s with *Chlorella* sp. for commercial purposes (Pantami *et al.*, 2020). Soon after, some countries such as USA and Mexico started to commercial cultivation of microalgae, involving other species such as *Spirulina* sp. Since then, microalgae and cyanobacteria have sparked a lot of interest due to their potential in various industries like biorefineries, biopharmaceuticals, food supplements, nutraceuticals, and other high-value products (Saeed *et al.*, 2022).

In aquaculture industry, microalgae generally known as a food source for marine organisms such as shrimp, molluscs and fish larvae. Spolaore *et al.* (2006) reported that in 1999, approximately one-fifth of the 5 000 tonnes of yearly global microalgae production were used in aquacultural hatcheries with molluscs as a main consumer with 62% followed by shrimp (21%) and another 16% were fish. Zmora *et al.* (2013) report that until 2011, total consumption of green water microalgae by cultured fish and crustaceans via planktivory was estimated to increase at 240 million tonnes. The increase in demand of microalgae by aquaculture industries show the important of microalgae in providing proteins and vitamins sources for aquaculture organisms (Muller-Feuga, 2013). Currently, more than 40 species of microalgae have been used as live or dead cell food sources to enhance the characteristics of aquatic animals, such as fish, mussels and shrimps (Rahman *et al.*, 2018).

Microalgae have many valuables nutraceutical substances such as carotenoids, phycocyanin and poly-unsaturated fatty acids (PUFAs). Demirel *et al.* (2009) and Guedes *et al.* (2011) reported in their study that microalgae extracts have been used in the cosmetics and pharmaceutical industries. It is commonly used as dietary supplements and recently being developed as anticancer agent. Microalgae contains compounds which found to play a major role in dealing with reactive oxygen species (ROS) and oxidative stress, which can cause multifactorial diseases such as cancer, cardiovascular diseases, and inflammatory disorders (Sachidanandam *et al.*, 2005; Laguerre *et al.*, 2007). The studies by Li *et al.* (2007) and Goiris *et al.* (2012) showed that microalgae are the good source of antioxidant compounds and were believed to have health benefits and useful in prevention of ageing related diseases and cancer.

In general, cancer can be highly related with damaged of the cell DNA as damaged DNA would occur in both the nuclear and mitochondrial genomes, which

lead to deleterious biological outcomes (Limpose *et al.*, 2014). Microalgae can be used as anticancer agents, by preventing DNA damage from occurring on human cell. Green microalgae such as *Tetraselmis suecica* contain active pigments such as carotenoids, which showed to have higher antioxidant and able to protect cells from DNA damage (Sansone *et. al.*, 2017). The author also observed that the green microalgae extracts can reduce prostaglandin E₂ (PGE₂) levels in the cells damaged by H₂O₂ and has tissue repairing effects on reconstructed human epidermal tissue cells (EpiDermTM).

Apart from that, oxidative stress on tissues is one of other factors that indirectly exposed the cell with DNA damage. Oxidative stress conditions are caused by endogenous oversized formation of reactive oxygen species (ROS) that exceeds the availability of antioxidants, which is caused by external stressors. The excessive ROS can cause damage especially to cellular proteins, polyunsaturated fatty acids, and DNA (Machu *et al.*, 2015). Theoretically, with the presence of polyphenols, which present abundantly in microalgae, the oxidative stress on tissue can be prevent. Polyphenol, a potent antioxidant serves as protecting agent for the body's tissues against oxidative stress (Miranda *et. al.*, 2008). As microalgae contain polyphenols compounds, they are good source of natural product in minimize the oxidative stress in cellular activities (Machu *et al.*, 2015). Although many studies have confirmed the antioxidant activities in microalgae (Yuan *et al.*, 2018), its effect (either the extract or compound) in prevention of DNA damage still scarce. Besides, most of studies related with biomedical and pharmaceutical industries usually focus on famously known species such as *Spirulina* sp. and *Chlorella* sp. Thus, I believed that other species of microalgae, *Nannochloropsis* sp. which generally known in aquaculture industry also have the ability to thwart the DNA damage in the cells due to its higher content in polyphenolic compounds. This research project is sought to determine the capability

of green microalgae, *Nannochloropsis* sp. extracts, in preventing DNA damage induced by hydrogen peroxide (H₂O₂) as the induction of DNA damage by H₂O₂ may mimicked the endogenous free radicals release in the body that can damage the DNA. Prevention of DNA damage is important as it can cause changes in the DNA sequence and thus can lead to cancer.

1.2 Objectives of the research project

1. To screen the chemical compounds in *Spirulina* sp. and *Nannochloropsis* sp. using High Performance Liquid Chromatography.
2. To determine phytochemical contents and evaluate antioxidant activities of *Spirulina* sp. and *Nannochloropsis* sp.
3. To evaluate the primary preventive effects of *Nannochloropsis* sp. extracts on the DNA damage, induced by hydrogen peroxide in normal cell line (L929).

CHAPTER 2

LITERATURE REVIEW

2.1 DNA Damage

2.1.1 Definition and cause of DNA Damage

DNA damage is one of biological process that usually occurred in living organisms. DNA damage can be defined as any modification in the physical and/or chemical structure of DNA that is capable of causing cellular injury and reduces viability or reproductive fitness of the organism (Xing, 2022). There are two main categories of DNA damage based on its sources which are endogenous and exogenous. The majority of the endogenous DNA damage arises from the chemically active DNA engaging in hydrolytic and oxidative reactions with water and reactive oxygen species (ROS), respectively, that are naturally present within cells (Chatterjee and Walker, 2017). ROS are type of unstable molecule that contains oxygen and that easily reacts with other molecules in a cell. A build-up of ROS in cells may cause damage to DNA, RNA, and proteins, and may cause cell death. ROS are free radicals also called oxygen radical. Study by Tropp (2011) reported that, the most conspicuous of the ROS species are the superoxide radicals ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\bullet\text{OH}$). There are two sources biological production of H_2O_2 which are produced in vivo through dismutation of superoxide radical ($\text{O}_2\bullet^-$), both non-enzymatically and catalysed by superoxide dismutase enzymes, the other one is directly produced by a range of oxidase enzymes including glycollate and monoamine oxidases as well as by the peroxisomal pathway for β -oxidation of fatty acids (Halliwell *et al.*, 2000). The danger of H_2O_2 largely comes from its ready conversion to the indiscriminately reactive hydroxyl radical ($\text{OH}\bullet$), either by exposure to ultraviolet light (Ichihashi *et al.*, 2003) or by interaction with a range of transition metal ions, of which the most

important *in vivo* is probably iron (Halliwell *et al.*, 2000). As endogenous DNA damage occur within the cells, the reactions of DNA with molecules from surroundings resulting in the development of hereditary diseases and sporadic cancers (Visconti and Grieco, 2009; Reuter *et al.*, 2010; Perrone *et al.*, 2016). On the other hand, exogenous DNA damage, occurs when environmental, physical and chemical agents damage the DNA. Examples include UV and ionizing radiation, alkylating agents, and crosslinking agents (Hoeijmakers, 2009).

Both main categories of DNA damage results in alteration of DNA that could occur up to 1×10^5 DNA lesion in a day involving pyrimidine dimers. Pyrimidine dimers which induced in DNA by ultraviolet (UV) perhaps is the generally known in DNA lesion that affecting a single DNA strand by blocking the progress of both DNA polymerases and RNA polymerases (Kuzminov, 2013). As pyrimidine dimers efficiently formed in denatured DNA than in duplex DNA, removal of these dimers is one of the most important DNA repair tasks of any cells with direct exposure to sunlight. The presence of cytosine at the unrepaired pyrimidine dimers will promote deamination which will cause replacement of cytosine with thymine, and thus changing the integrity of the DNA (Schuch *et al.*, 2017). The long exposure to radiation and chemical from various sources, endogenous reactive oxygen species and other biological processes in the body such as hydrolysis and alkylation damaging the DNA sequence by breaking the hydrogen bond that strongly hold the two single strands of DNA forming double stranded helical structure of DNA. If this damage is not being repair correctly, the re-joining of single stranded DNA could cause error, shifted in DNA sequence and greatly induced mutation and cancer (Lord and Ashworth, 2012). Besides that, Kastan (2008) in his study mentioned DNA damage is well known fact that causes cancer since *in vitro*, *in vivo* studies, clinical studies on human exposed

to radiation environment and genetic studies on genes involved in DNA damage responses proved that DNA damage leads to cancer. DNA damage were believed to cooperate well with cancerous cells compare to non-cancerous where combination between cancerous cells and DNA damage could develop resistance towards conventional cancer treatment besides facilitate the carcinogenesis process (Liang *et al.*, 2009). The illustration on how the internal and external sources caused cancer are summarized in Figure 2.1.

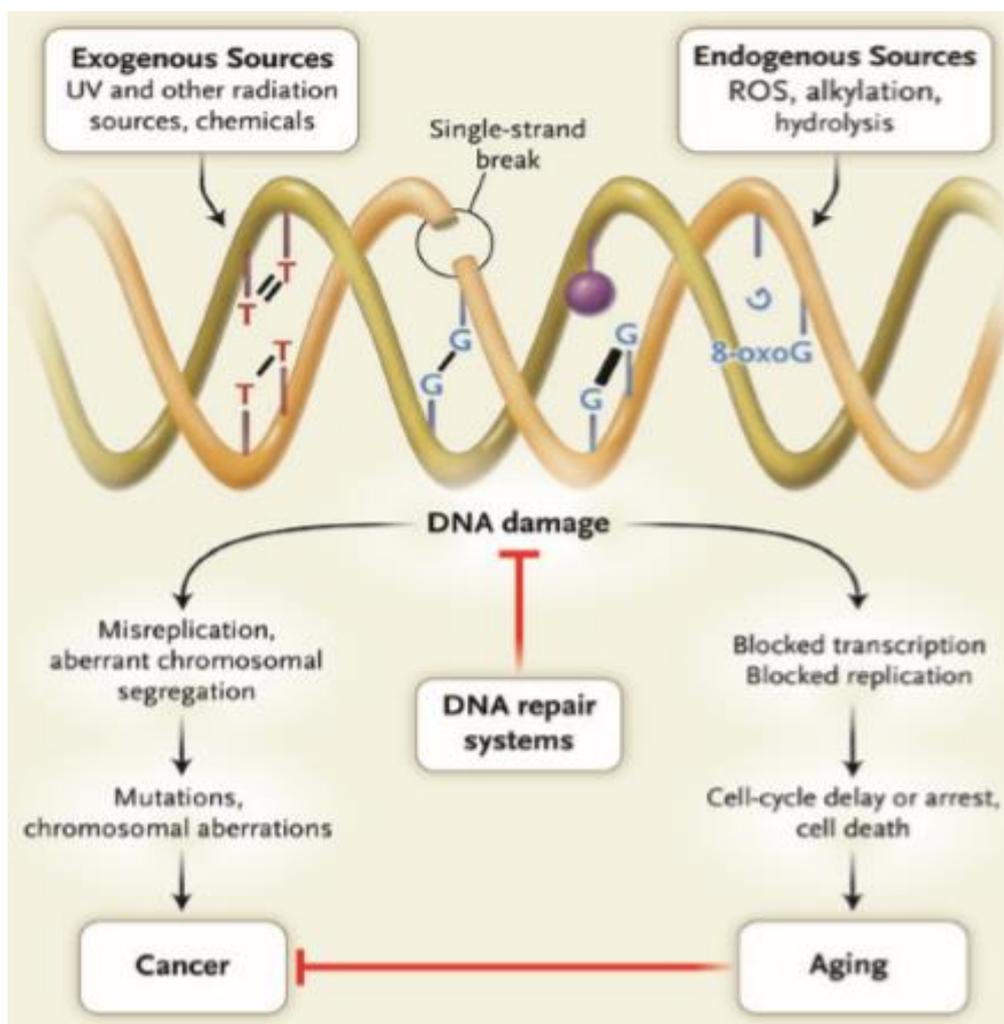


Figure 2.1: The simple illustration shows how exogenous sources and endogenous sources contribute in alteration of DNA. As the DNA repair systems failed to work normally, the cell that replicate might carry the abnormal chromosomes and mutations permanently (Hoeijmakers, 2009)

2.1.2 DNA Damage repair mechanism

As the DNA of a cell were damaged, genomic stability had been altered. To overcome the problem thus maintaining their genome stability, evolving process of cellular responses were occurred in eukaryotes toward DNA damage which led to preventing from cancer initiation and progression (Synowiec *et al.*, 2008; Pardo *et al.*, 2009). Through the evolved cellular responses, cells were enabled to either cope or eliminated the damage or to activate process of programmed cell death when the damage could not be repaired, thus preventing any mutations were passed to the progeny cells (Sancar *et al.*, 2004; Maynard *et al.*, 2009). Generally, there are five different pathways to repair the damaged DNA as the pathway depends on the source of the damaged. Direct Repair (DR) for damage caused by O6Alkyl Guanine, Base Excision Repair (BER) for repairing damaged base such as deaminated bases, oxidized-reduced or alkylated, Nucleotide Excision Repair (NER) for bulky DNA lesions, Mismatch Repair (MMR) for the replacement of mismatched bases and lastly Double Strand Break Repair (DSBR) which repair breaking of DNA double-strand. All the pathways were stimulated based on lesion induced from DNA damage.

2.1.2 (a) Direct Repair (DR)

Eker *et al.* (2009) in their study reported that DR normally known as simplest repair pathways which involving single protein that remove lesion in error-free manner without effecting sugar-phosphate backbone or the base excision itself. The study also described two main class of DR which are repair of alkylating damage by alkyltransferase and dioxygenases and the other one, repair of UV light-induced photolesions by spore photoproduct lyases and photolyases.

2.1.2 (b) Base Excision Repair (BER)

This pathway divided into two minor class which are short-patch and long-patch (Robertson *et al.*, 2009; Davis and Lin, 2011). BER repairs the damaged DNA by correcting base lesion that induced by oxidation, deamination, depurination/depyrimidation and alkylation (Robertson *et al.*, 2009). BER were believed to play major role in repairing damaged DNA from endogenous sources (Hoeijmakers, 2001). Besides that, BER are important in repairing single strand brake (SSB) as BER not only repairing SSB but it also can identify and eliminate the altered chemical bases such as abasic site, methylation sites and uracil incorporation (Martin, 2001). Elimination of abasic site that also known as apurinic/aprimidinic (AP) sites are crucial as they are one of the most frequent spontaneous lesions in DNA which potentially mutagenic and lethal lesions that can block DNA replication and transcription that results in generating DNA single-strand breaks (SSBs) with 5'- or 3'-blocked ends (Boiteux and Guillet, 2004). Apart from that, repairing methylation sites of DNA are important as methylation of DNA is common epigenetic signalling tool that cells use to lock genes in the "off" position (Phillips, 2008). Catalysed by a family of DNA methyltransferases, majority of methylation occurs in cytosines that precede guanine nucleotide or CpG sites (Moore and Fan, 2013). Uracil incorporation is situation where uracil is form from incorporation of dUMP during replication and from spontaneous or enzymatic deamination of cytosine, resulting in U:A pairs or U:G mismatches (Visnes *et al.*, 2009). Even though uracil incorporation is not mutagenic towards DNA, it can influence genomic stability which can leads toward DNA damage (Chakraborty and Stover, 2020). Thus, BER is a crucial process in preventing mutagenesis from occurred (Hoeijmakers, 2001).

2.1.2 (c) Nucleotide Excision Repair (NER)

NER pathway is complicated compared to DR and BER since the damaged base will be identified and removed before DNA polymerase fills the gap and rejoined through ligase activity (Davis and Lin, 2011). NER is mostly involved in the repair process caused by both endogenous and exogenous sources of DNA damage such as UV radiation, chemical, protein addition and ROS (Sancar *et al.*, 2004; Maynard *et al.*, 2009). Similar to BER, NER is categorized into two which are transcription-coupled repair (TCR) and global genome NER (GG-NER) (Hanawalt, 2002).

2.1.2 (d) Mismatch Repair (MMR)

Based on the name itself, this pathway takes place by the elimination of insertion-deletion loops and mismatched bases generated during replication of DNA. By undergoing this process, MMR pathways which are reported to be linked with BER and NER pathway components, are able to prevent mutation and conserve genomic stability of cells (Zhu *et al.*, 2009).

2.1.2 (e) Double Strand Break Repair (DSBR)

As we mentioned earlier, Double Strand Break (DSB) can be damaged or induced by multiple sources of endogenous and exogenous factors such as chemically active DNA engaging in hydrolytic and oxidative reactions with water, ROS, environmental, physical and chemical agents damage the DNA, UV and ionizing radiation, alkylating agents, and crosslinking agents. As there are widely known sources that cause DSB, it has been known as the most lethal among the others. Rich *et al.* (2000) mentioned that a single DSB can lead to cell death. To overcome this major problem, DSBR in humans consists of two routes which are homologous recombination (HR) and non-homologous end-joining pathway (NHEJ). Many studies have been carried out by researchers on DSBR and one of

the studies conducted by Branzei and Foiani (2008) reported that during cell cycles including S phase, HR and NHEJ might be competed with each other. In contrary, Kim *et al.* (2005) mentioned that there is no competing between HR and NHEJ as both of the pathways are closely related to each other. As both of the pathways actively involved in cell cycle, NHEJ were believed to be more powerful pathway compare to HR since NHEJ are able to simply repair the DSB thru re-ligating any kind of DSB without presence of sister chromatids (Pardo *et al.*, 2009). Wang *et al.* (2013), in his study proof that HR were inactive in G0/G1 phase due to lacking of homologous sister chromatid.

2.2 Natural product role in DNA Damage

Natural products were identified as chemical substances or compounds that produced by living organism that having biological or pharmalogical properties (Gollahon *et al.*, 2011). Based on data produced by World Health Organization (2022), 88% of all countries are estimated to use natural products as traditional medicine, such as herbal medicines, acupuncture, yoga, indigenous therapies and others. Natural product starts to emerge in medical field as increasing demand in industry due to the affordable, safety and efficacy properties compare to synthetic products (Topliss *et al.*, 2002).

In the present, pharmaceutical properties in natural products shown the potency of natural product itself as sources for drug discovery. Chin *et al.* (2006) reported that, 23 new drugs were derived from natural product between 2000 to 2005 that can be used in various diseases such cancer, cardiovascular, metabolic disease and others.

Natural products also known as natural drugs play major role in repairing DNA damage pathway since the drugs act even as a biological catalyst where the rate of the

repair process is enhanced (Pillai *et al.*, 2010). The rate of repair process is crucial in order to maintain the genomic stability of cell. Pillai *et al.* (2019) mentioned that reducing rate of lifespan and increasing cancer incidence has been observed in experimental animals that have genetic deficiencies in DNA repair. There are few natural products which showing positive characteristic in DNA protection such as phenolic compounds, essential oils, alkaloids, carotenoids, glutathione and glucans. Generally, most of all the natural products can be categorised as antioxidant. Antioxidant were told to have close relationship with DNA damage somehow because majority of compound that proof to be preventive and repairing agent in DNA damage showed antioxidant capacity (Pillai *et al.*, 2019).

2.2.1 Antioxidant as preventive aspect on DNA damage

Among the sources that induced DNA damage on cell, oxidative DNA damage is considered as the most common insult which resulting in increasing H₂O₂ (Li *et al.*, 2020). However, presence of antioxidant makes a way to overcome the excessive oxidative DNA. Antioxidants defined as molecules that inhibit or quench free radical reactions and delay or inhibit cellular damage (Young and Woodside, 2001). Antioxidants generally can be categorised into various ways but in view of DNA damage, antioxidant mainly divide into two types based on their activity which are enzymatic and non-enzymatic antioxidant (Nimse and Pal, 2015). Enzymatic antioxidant is complex compare to non-enzymatic antioxidant since enzymatic antioxidant reaction involved in complex process with presence of cofactors such as copper, zinc and iron in order to changed oxidative products to H₂O₂ before turned it into water. In other hand, non-enzymatic antioxidant such as vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione (Shahidi and Zhong, 2010) easily interacts with oxidative product by interrupting free radical chain reaction. Besides

that, the size of antioxidant itself determined the way it react with oxidative products. For small-molecule antioxidant, they undergo process called radical scavenging of the ROS in order to neutralize before carry them away. Contrary with large-molecule antioxidant which usually in form of enzymes (SOD, CAT, and GSHPx) and sacrificial proteins (albumin), they react by absorbing the ROS itself to prevent it from attacking other essential proteins (Nimse and Pal, 2015).

Presence of antioxidant in cellular activity and maintaining the level itself are crucial key in order to lower the risk of getting disease. Pal *et al.*, (2012) in their study showed that lack of antioxidant has been associated with heart disease and cancer. Besides that, antioxidant also shown protective effect against number of diseases such as aging, allergies, algesia, arthritis, asthma, atherosclerosis, autoimmune diseases, cancer, bronchopulmonary dyspepsia (Nimse and Pal, 2015) cataract, cerebral ischemia, diabetes mellitus, eczema, gastrointestinal inflammatory diseases and genetic disorders (Lissi *et al.*, 1995).

There are several antioxidant defences in all organisms to protect themselves against hostile oxidative environment. The defences include the classical antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase as well as the non-enzymatic reactive oxygen species (ROS) scavengers, including vitamin C and E (Espinosa-Diez *et al.*, 2015). Glutathione peroxidase was reported as the robust biological antioxidative reductant among all the antioxidant enzymes (Cross *et al.*, 1977). It is important to sustain a good condition of redox balance status for the physiological acid-base buffer system in the body so that the homeostatic cellular activities will maintain at the optimum level. Since most of the activation and reaction in the body is relying on the reduction-oxidation processes, the alteration in redox

balance would give a great impact on the signalling of cellular pathway and the transcriptional activities (Tan *et al.*, 2018).

2.3 Microalgae as source of antioxidant

Microalgae is one of the natural products that has been widely used as a natural supplement to maintain a good health in human. It was used by indigenous populations for centuries, but the microalgal biotechnology only really began to develop in the middle of last century (Spolaore *et al.*, 2006). Microalgae also known as one of the new model organisms with many potential beneficial uses in biotechnology application involving the nutritional supplement to human and animal, the production of biodiesel (Marques *et al.*, 2019) and bioremediation of wastewater (Spolaore *et al.*, 2006). They can be found in two habitat; marine or freshwater (Shahid *et al.*, 2019). In Figure 2.2, difference of microalgae can be identified based on the morphology and cellular colouration.

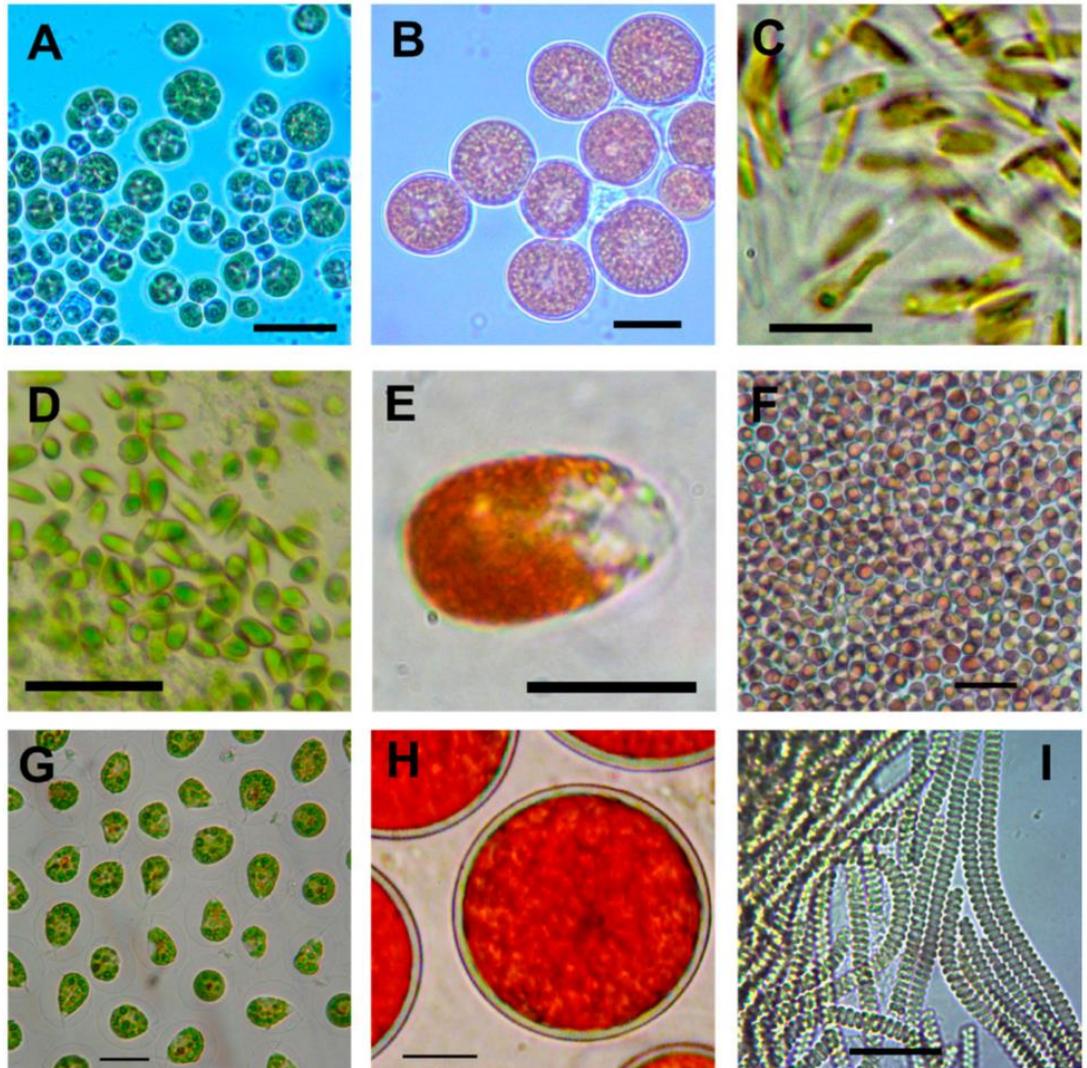


Figure 2.2: The light micrographs of morphology and cellular colouration of microalgae with high potential in various biotechnology applications. *Chlorococcum* sp. ((A,B); carotenoids source including astaxanthin, β -carotene, canthaxanthin and lutein), *Dunaliella salina* (C), *Phaeodactylum tricornutum* (essential fatty acid, EPA, source), ((D,E); β -carotene source) and *Haematococcus pluvialis* (F), *Porphyridium cruentum* (natural pink colourant phycoerythrin and bioactive polysaccharides) ((G,H); astaxanthin source) are cultivated as two distinct growth phases: (1) green-phase ((A,D,G); for biomass generation) and (2) stress-phase ((B,E,H); for carotenoids and fatty acids accumulation). and (I), *Arthrospira* (*Spirulina*) sp. (natural blue colourant phycocyanin and multiple health benefitting ingredients) are cultivated as single-phase actively growing biomass for the targeted biomolecules. All scale bars are 25 μm , except for (C, E), which are 10 μm (Saha and Murray, 2018)

The blue-green algae such as *Nostoc*, *Spirulina* and *Aphanizomenon* species has been used as food for thousands of years. This type of microalgae was classified as the most primitive life forms on earth with a simple prokaryote cellular structure (Jensen, 2001). The author also mentions that blue-green algae also having a characteristic of animal kingdom, in which containing cellular membrane complex sugars similar to glycogen. The unicellular microalgae constitute of structure with small size as 0.2 micrometre while the multi-cellular macroalgae have a length up to 60 metres, and both are photosynthetic species (Christaki *et al.*, 2011). Normally, the present of microalgae are not observable by naked eye, but the algal growth massively, it can cause the changing of the water in a green, brown, blue or orange liquid mass depending on the types of the algae. The estimated varieties number of microalgae are very diverse which ranging from 200,000 to 800,000 algae species (Wolkers *et al.*, 2011).

A global trend to replace chemical antioxidants with natural antioxidants has also been in place over the past twenty years (Safafar *et al.*, 2015) and algae have been at the middle of interest in recent years as a rich source of bioactive compounds such as phenolic compounds, fatty acids, amino acids, and carotenoids. It has been a good source of antioxidant compounds and act as a natural source for pigments, proteins, lipids and vitamins such as Vitamin A, B1, B2, B6, B12, C and E as well as minerals such as iodine, calcium, magnesium, iron and potassium (Becker, 2013). Thus, it has the capability to prevent and cure various acute and chronic diseases. Figure 2.3 demonstrates the various product that can be extracted from microalgae biomass via biorefinery approach which useful for human health supplementation. In the current state, the microalgae such as *Schizochytrium*, *Dunaliella*, *P. cruentum*, *Spirulina*, *Chlorella*, *Haematococcus*, and *C. cohnii* are commonly been farmed, harvested and

extracted for human usage as food supplement since these species are classified as food sources falling into the Generally Recognized as Safe (GRAS) category by Food and Drug Administration, FDA. Whereas, certain microalgae such as *Nannochloropsis* sp. were mainly farm to generate oil for biodiesel (Ma *et al.*, 2014).

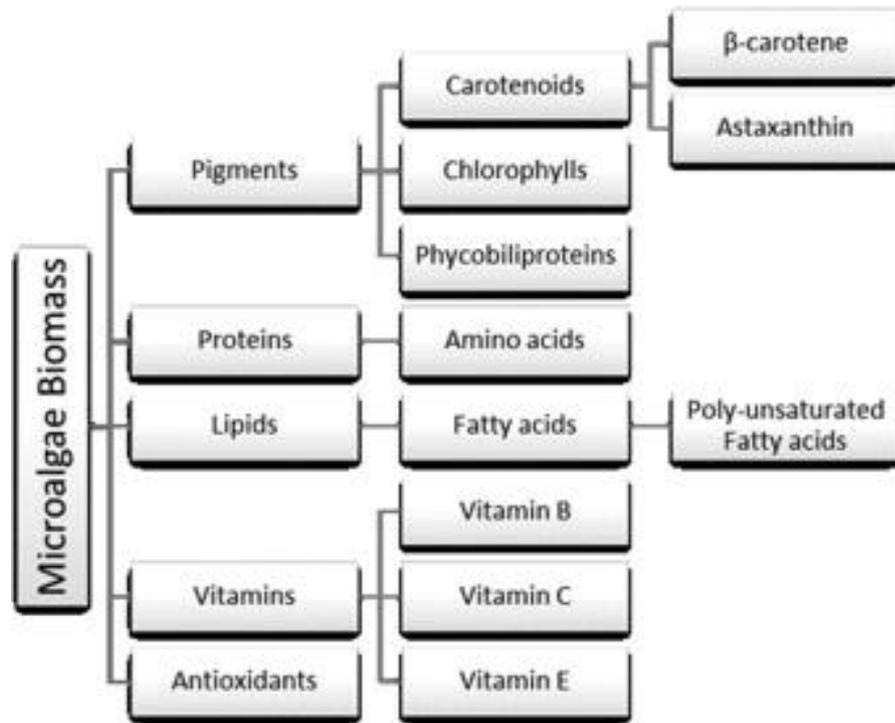


Figure 2.3: Various microalgae biomass extracted by biorefinery approach and produce the end products for health supplements (Koyande *et al.*, 2019).

2.3.1 *Spirulina* sp.

Spirulina (*Arthrospira*) is one of the microalgae which was reported of having rich sources of vitamins with 62% of amino acid as well as carotenoids and xanthophyll. It has gained attention in the worldwide market as a food supplement due to the high protein content and the excellent nutrient value (Sathasivam *et al.*, 2019). Moreover, the content of essential fatty acids and linolenic acid which cannot be naturally produced by humans body can be supplied to consumer by taking this type of microalgae (Becker, 2007). Studies had shown that microalgae could produce

polyphenol such as p-coumaric acid, the precursor of flavonoid synthesis (Goiris *et al.*, 2014). Besides that, HPLC analysis of *Spirulina maxima* showed the present of flavonoids and phenolic acids such as gallate, chlorogenate, cinnamate, pinostrobrate and p-OH-benzoates (Abd El-Baky *et al.*, 2009) which shown to be hepatoprotective with strong antioxidants activity. Apart from that, flavonoids and phenolic acids previously shown to exert the anti-allergic, anti-inflammatory, anti-diabetic, anti-microbial, anti-pathogenic, anti-viral, anti-thrombotic, and vasodilatory effects (Huyut *et al.*, 2017). The author also mentioned that both flavonoids and phenolic acids can prevent health disorders including Alzheimer's, cancer, heart problems, cataracts and eye problems. The strong effects of antioxidant were believed to inhibit the oxidation of lipid which capable to protect against oxidative stress diseases (Ayala *et al.*, 2014). In addition, flavonoids also play important role in blocking lipoxygenase enzyme activities (Pietta, 2000) which crucial in order to prevent inflammation-related diseases such as cancer (Wisastra and Dekker, 2014).

Jadaun *et al.* (2018) in their study shown that *Spirulina* extract used was effective in preventing all the enhanced oxidative stress effects. In the study, H9c2 was cultured under high glucose level (33mM) which cause increasing in oxidative stress besides malfunctioned the mitochondria membrane of the cell. By introducing *Spirulina* extract to the cell, the level of ROS was repressed and ability of mitochondria membrane were enhanced. They concluded that the extract from *Spirulina* functioned well in preventing the oxidative stress even though the condition is very susceptible to DNA damage as well as related dysfunction in H9c2 cells.

2.3.2 *Nannochloropsis* sp.

Nannochloropsis represents a genus of marine microalgae with high photosynthetic efficiency which converting carbon dioxide to storage lipids mainly in the form of triacylglycerols and to the omega-3 long-chain polyunsaturated fatty acid eicosapentaenoic acid (EPA) (Ma *et al.*, 2016). This green microalgae has capability to produce high concentration of important antioxidant such as zeaxanthin and canthaxanthin, which are valuable in nutraceuticals and pharmaceutical industries (Asker *et al.*, 2018). The risk of age-related macular degeneration and few eye disorders were reported can be reduced by consuming this microalgae, due to the high content of zeaxanthin (Mares, 2016). Both zeaxanthin and canthaxanthin are useful in slowing the atherosclerosis process by inhibiting low-density lipoprotein oxidation (Carpenter *et al.*, 1997). Besides that, Chew *et al.* (1998) reported in their study that canthaxanthin was previously showed to inhibit the growth of mammary tumour in mice in dose-dependent manner.

Study carried out by Letsiou *et al.*, (2017) on the effects of *Nannochloropsis gaditana* extract against oxidative stress (induce by hydrogen peroxidase, H₂O₂) in human primary fibroblasts reported that the extract of the microalgae exhibits skin protection properties by preventing the oxidative responses which cause cell death.

2.3.3 Microalgae Chemical Contents

The chemical contents in microalgae are vary from species to species and thus it can be used to differentiate certain species of microalgae and grouping the microalgae into few major groups (Wright and Jeffrey, 2006). For example, the high level of chlorophylls in cyanobacteria species can be used to differentiate it with *Glaucophytes* sp., which having various type of carotenoids.

Carotenoids shows a great variety deals of pigments found on earth. They are a lipophilic compounds which usually can be seen in yellow, red or orange colours (Varela *et al.*, 2015). Most of the carotenoids having different number of carbon-based backbone structure. The structure of the backbone with isoprene units called terpenoid are further classified into carotenes and xanthophyll. The common carotenoid's structure found in microalgae are lycopene, β -carotene, lutein, zeaxanthin, canthaxanthin, and astaxanthin (Sathasivam and Ki, 2018). These compounds were reported to have many health benefits. Astaxanthin have been shown to have a very strong antioxidant property (Ebrahimzadeh *et al.*, 2017), anti-inflammatory effects (Davinelli *et al.*, 2018), anti-cancer and help in cardiovascular health (Visioli and Artaria, 2017). Astaxanthin is the strongest antioxidant in the group of carotenoids, in which it was shown to have more than two-fold of antioxidant when compared to β -carotene and vitamin E (Li *et al.*, 2011). Other carotenoids such as lutein and β -carotene were reported to prevent cataract (Manayi *et al.*, 2015) and night blindness (Dufosse *et al.*, 2005). These bio-product becoming an important supplements in-demand in the nutraceutical market since they are playing a vital role in maintaining eye health. Apart from that, Lycopene, a non-provitamin A carotenoid involves in wide range of biological activities. It was able to prevent prostate cancer and was used as supplement in the treatment for cardiovascular diseases (Zhang *et al.*, 2015).

Other than carotenoids and chlorophylls, phycobiliprotein also one of the components in light harvesting pigment family complexes found in microalgae (Stanic-Vucinic *et al.*, 2018). It is having high-value natural product with potential in industrial and day to day applications. Commercially, phycobiliprotein is being used as non-harmful natural food colorant replacing the synthetic colorant which was probably known to have carcinogenic and toxic potential to consumers. Moreover, this

type of pigments also has been extensively used as fluorescent applications in clinical and immunological analysis (Sekar and Chandramohan, 2008).

Protein is one of the important nutritional components in microalgae. There are number of microalgae species which were reported to contain approximately the same amount of protein as compared to the main protein sources such as meat, milk, soybean and egg. The protein production yield from microalgae is way more efficient as compared to terrestrial crops. Up to 15 tons of protein yield was extracted from microalgae per year than 1 to 2 tons per year from wheat, soybean and pulse legumes (Krimpen *et al.*, 2013). The example of microalgae which rich in protein content are *Nostoc sp.* and *Arthrospira sp.* (usually denoted as *Spirulina* in the market), and *Aphanizomenon sp.* When it comes to quality, *Chlorella* and *Arthrospira* accumulate high-quality proteins, having both species a well-balanced amino acid profiles according to the WHO/FAO/UNU recommendations in regard to human requirements of essential amino acids (Becker, 2007).

The other important contents of microalgae are lipids, in the form of a long-chain polyunsaturated omega-3 fatty acids. The main rich-nutrient compounds that can be obtain from microalgae-based lipids are eicosapentaenoic acid (EPA), α -linolenic acid (ALA), docosahexaenoic acid (DHA) and stearidonic acid (SDA) (Wells *et al.*, 2017). Normally, terrestrial plants are the main producer and having high amount of ALA but the long chain EPA and DHA only can be obtained from aquatic organisms (Hixson *et al.*, 2015). In the market, EPA and DHA are mainly sources from fish and since human body incapables to synthesis both EPA and DHA, this omega 3 are considered as an essential dietary supplement to be consumed. At commercial level, EPA and DHA are the popular supplements in cardiovascular diseases prevention and treatments (Ryckebosch *et al.*, 2012). The use of EPA and DHA supplement in

Alzheimer's patients also showed a great results as these patient had been showed clinically to have low level of DHA and the consumption of EPA and DHA not only rectify the low DHA level, but give the good progress in term of cognitive function in patient with very mild Alzheimer's symptom (Swanson *et al.*, 2012).

Apart from that, secondary metabolites such as flavonoids were reported present in quantities in microalgae species. Study by (Goiris *et al.*, 2014) showed the present of ferulic acid and *p*-Coumaric acid in microalgae species that they were screened. Not only ferulic acid and *p*. coumaric acid were found in microalgae, Onofrejev *et al.* (2010) in their study reported that caffeic acid was also present in some species of microalgae in small quantities. These compounds were shown to posses strong antioxidant activities which protective to oxidative-DNA damage (Zdunska *et al.*, 2018; Boo, 2019)

CHAPTER 3

MATERIALS AND METHODS

This chapter discussed on the material and methods involved in this study. Methods have been divided into three parts; part I: Sample analysis, II: Phytochemistry study and Part II: DNA Damage study. The works were conducted in the laboratories of Integrative Medicine Cluster, Oncology Cluster and Regenerative medicine Cluster, Advanced Medical Dental Institute (AMDI), USM. Table 3.1 and Table 3.2 below showed the reagents and materials used in this study.

Table 3.1: Reagents and chemicals.

Study	Material and reagent	Supplier
Sample analysis	Methanol HPLC grade Acetonitrile HPLC grade Formic acid	Fisher Scientific, New Hampshire, United States America
	Caffeic acid Kaempferol P- coumaric acid Hesperetin Myricetin Balcaline Quercetin Naringenin	Sigma Aldrich, Missouri, United States America

Phytochemistry study	2,2-diphenyl-1-picrylhydrazyl (DPPH) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) Quercetin Trolox	Sigma Aldrich, Missouri, United States America
	Gallic acid Folin – Ciocalteu reagent Sodium hydroxide Sodium nitrate Sodium carbonate Aluminium chloride	Merck, New Jersey, United States America
	Methanol AR grade Ethanol AR grade	QRec, New Zealand
DNA Damage study	Comet Assay Kit i. Lysis solution ii. LMAgarose iii. Alkaline unwinding solution iv. Alkaline electrophoresis solution v. EDTA	Trevigen, Gaithersburg, United States America
	Tris	Invitrogen, California,