ANTIOXIDANT CAPACITIES AND BIOACTIVE COMPOUNDS OF
NOVEL THERMO-TOLERANT GREEN MICROALGAE

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submitted by **ONUR BULUT** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Biochemistry, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences**   _____

Assoc. Prof. Dr. Yeşim Soyer
Head of the Department, **Biochemistry**   _____

Prof. Dr. Hüseyin Avni Öktem
Supervisor, **Biological Sciences, METU**   _____

Assoc. Prof. Dr. Mahmut Deniz Yılmaz
Co-Supervisor, **Bioengineering,
Konya Food and Agriculture University**   _____

Examining Committee Members:

Prof. Dr. Füsun Eyidoğan
Faculty of Education, Başkent University   _____

Prof. Dr. Hüseyin Avni Öktem
Biological Sciences, METU   _____

Assoc. Prof. Dr. Çağdaş Devrim Son
Biological Sciences, METU   _____

Assoc. Prof. Dr. Çağla Sönmez
Molecular Biology and Genetics,
Konya Food and Agriculture University   _____

Assoc Prof. Dr. Deniz Çekmecelioğlu
Food Eng., METU   _____

Date: 04.08.2020

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Name, Last name : Onur Bulut

Signature :

ABSTRACT

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Bulut, Onur
Doctor of Philosophy, Biochemistry
Supervisor: Prof. Dr. Hüseyin Avni Öktem
Co-Supervisor: Assoc. Prof. Dr. Mahmut Deniz Yılmaz

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Accumulation of reactive oxygen species (ROS) at high levels in cells as a result of normal cellular activities as well as exposure to oxidative stress is attributed to many chronic degenerative diseases and conditions in humans including cardiovascular diseases, cancer and aging-related conditions such as Alzheimer's disease. Although living organisms are equipped with enzymatic and non-enzymatic antioxidant defense systems to counteract the adverse effects of oxidative stress, yet they rely on exogenous sources of antioxidants for additional support. Despite, fruits and vegetables are the predominant sources, alternative sources of antioxidants such as microalgae are also being explored.

In present study, novel thermo-tolerant green microalgal strains, *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05, were evaluated for their antioxidant capacities, total phenolic, flavonoid and carotenoid contents in various solvent extracts. Ethanol/water extracts of *Hindakia tetrachotoma* ME03 had the highest total phenolic content with 18.30 ± 0.72 mg gallic acid equivalents g^{-1} DW and displayed the strongest antioxidant activity; 12.42 ± 1.21 and 67.98 ± 3.45

μmol trolox equivalents g^{-1} DW measured by DPPH and FRAP assays, respectively. Although solvent extracts of *Micractinium* sp. ME05 showed relatively lower antioxidant capacity, they had the highest total flavonoid content (5.72 ± 0.26 mg quercetin equivalents g^{-1} DW) and carotenoid content (3.17 ± 0.21 mg g^{-1} DW).

Moreover, twelve different phenolic compounds were analyzed and quantified in different solvent extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 by reversed phase-HPLC. Ethyl acetate extract of *Scenedesmus* sp. ME02 showed substantial amounts of quercetin (0.84 ± 0.12 mg g^{-1} DW) and rutin (0.11 ± 0.08 mg g^{-1} DW). Solvent extracts of *Micractinium* sp. ME05 grown under mixotrophic conditions were determined to have high amounts of gallic acid (0.46 ± 0.15 mg g^{-1} DW) and rutin (0.21 ± 0.12 mg g^{-1} DW); whereas heterotrophic samples contained high amounts of gallic acid (0.12 ± 0.01 mg g^{-1} DW), benzoic acid (0.10 ± 0.01 mg g^{-1} DW), 4-hydroxy benzoic acid (0.40 ± 0.02 mg g^{-1} DW) and cinnamic acid (0.19 ± 0.06 mg g^{-1} DW).

Ethanol/water extracts of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03, and methanol extract of *Micractinium* sp. ME05 were further evaluated for their cytotoxic effects, inhibitory effects on intracellular ROS generation and cytoprotective effects. None of the microalgal extracts showed cytotoxicity on HeLa and MCF-7 cells. In contrast, the solvent extracts significantly inhibited H_2O_2-induced intracellular ROS generation in a concentration-dependent manner. At the highest concentration of 400 μg mL^{-1}, ethanol/water extracts of *Hindakia tetrachotoma* ME03 inhibited the intracellular ROS production by 76.11 ± 0.17% in MFC-7 cells, which was followed by methanol extracts of *Micractinium* sp. ME05 (72.62 ± 2.50%) and ethanol/water extracts *Scenedesmus* sp. ME02 (66.83 ± 5.50%). In consistent with their potential antioxidant capacities, the microalgal extracts also reduced H_2O_2-induced apoptosis and necrosis in MCF-7 cells. In comparison to the control group (54.83 ± 3.87%), the viability of MCF-7 cells pretreated with *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05

extracts significantly increased to 69.86 ± 0.25%, 86.6 ± 2.55% and 74.01 ± 2.32%, respectively.

This is the first study that reports significant amounts of phenolic compounds in *Scenedesmus*, *Hindakia* and *Micractinium* species. Moreover, this is the first time that the antioxidant capacity of *Hindakia* and *Micractinium* species is reported and the bioactive content of two different cultivation modes has been compared.

Keywords: Green Microalgae, Oxidative Stress, Antioxidant Capacity, Bioactive Compounds, Cytoprotective Activity

ÖZ

ISIL DİRENÇLİ YEŞİL MİKROALGLERİN
ANTİOKSİDAN KAPASİTESİ VE BİYOAKTİF BİLEŞİKLERİ

Bulut, Onur
Doktora, Biyokimya
Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem
Ortak Tez Yöneticisi: Doç. Dr. Mahmut Deniz Yılmaz

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Reaktif oksijen türlerinin (ROT) hücresel aktivite ve oksidatif stres sonucunda hücre içerisinde yüksek miktarlarda birikmesi, kardiyovasküler hastalıklar, kanser ve Alzheimer hastalığı gibi yaşa bağlı birçok kronik dejeneratif hastalıkla ilişklidir. Canlı organizmalar oksidatif stresin etkileri ile baş edebilmek için enzimatik ve enzimatik olmayan antioksidan savunma sistemlerine sahip olmasına rağmen yine de dış kaynaklı antioksidanlara ihtiyaç duymaktadır. Meyve ve sebzeler en etkili antioksidan kaynağı olmasına karşın mikroalgler gibi alternatif antioksidan kaynakları da araştırılmaktadır.

Bu çalışmada, üç farklı ısıl dirençli yeşil mikroalg türünden elde edilen solvent ekstraklarının (*Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 ve *Micractinium* sp. ME05) antioksidan kapasiteleri, toplam fenolik, flavonoid ve karoten içerikleri araştırılmıştır. *Hindakia tetrachotoma* ME03 türünün etanol/su ekstraktının en yüksek toplam fenolik içeriğe sahip olduğu (18.30 ± 0.72 mg gallik asit eşdeğeri g^{-1} KA) ve en yüksek antioksidan aktiviteyi gösterdiği belirlenmiştir. DPPH ve FRAP analizlerine göre toplam antioksidan aktivitenin sırasıyla 12.42 ± 1.21 ve 67.98 ± 3.45 µmol troloks eşdeğeri g^{-1} KA olduğu tespit edilmiştir.

Micractinium sp. ME05 türünün solvent ekstraktları daha düşük antioksidan aktivite göstermiş olsa da, en yüksek toplam flavonoid içeriğe (5.72 ± 0.26 mg kuversetin eşdeğeri g^{-1} KA) ve karoten içeriğe (3.17 ± 0.21 mg g^{-1} KA) sahip olduğu belirlenmiştir.

Ayrıca, *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 ve *Micractinium* sp. ME05 türlerinin farklı solvent ekstraktlarında on iki farklı fenolik bileşik ters fazlı HPLC ile analiz edilmiştir. *Scenedesmus* sp. ME02 türünün etil asetat ekstraktlarında oldukça yüksek miktarda kuversetin (0.84 ± 0.12 mg g^{-1} KA) ve rutin (0.11 ± 0.08 mg g^{-1} KA) belirlenmiştir. Miksotrofik koşullar altında büyütülen *Micractinium* sp. ME05 türünün solvent ekstraktları yüksek miktarda gallik asit (0.46 ± 0.15 mg g^{-1} KA) ve rutin (0.21 ± 0.12 mg g^{-1} KA) içermektedir; heterotrofik örneklerin ise yüksek oranda gallik asit (0.12 ± 0.01 mg g^{-1} KA), benzoik asit (0.10 ± 0.01 mg g^{-1} KA), 4-hidroksil benzoik asit (0.40 ± 0.02 mg g^{-1} KA) ve sinnamik asit (0.19 ± 0.06 mg g^{-1} KA) içerdiği tespit edilmiştir.

Scenedesmus sp. ME02 ve *Hindakia tetrachotoma* ME03 türlerinin etanol/su ekstraktlarının ve de *Micractinium* sp. ME05 türünün metanol ekstraktlarının yüksek antioksidan kapasitesi ve biyoaktif bileşik içeriğinden dolayı sitotoksik etkileri, hücreiçi ROT oluşumu üzerine etkileri ve hücre koruyucu etkileri araştırılmıştır. Mikroalg ekstraktlarının hiçbiri HeLa ve MCF-7 hücrelerine karşı toksik bir etki göstermemiştir. Aksine, bu ekstraktlar H_2O_2 ile indüklenen hücreiçi ROT üretimini konsantrasyonlarına bağlı olarak ciddi derecede inhibe etmiştir. En yüksek konsantrasyonda (400 µg mL^{-1}), *Hindakia tetrachotoma* ME03 türünün etanol/su ekstraktlarının MCF-7 hücrelerinde hücreiçi ROT üretimini %76.11 ± 0.17 oranında azalttığı belirlenmiştir. Arkasından ise %72.62 ± 2.50 ve %66.83 ± 5.50 inhibisyon oranları ile *Micractinium* sp. ME05 ve *Scenedesmus* sp. ME02 ekstraktarı gelmektedir. Antioksidan kapasiteleri ile ilişkili olarak, mikroalg ekstraktları MCF-7 hücrelerinde H_2O_2 ile indüklenen apoptoz ve nekroz üzerinde de etki göstermiştir. Sadece H_2O_2 uygulanan kontrol grubu (%54.83 ± 3.87) ile kıyaslandığında, *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 ve *Micractinium* sp. ME05

ekstraktları uygulanan MCF-7 hücrelerinin canlılık oranının sırasıyla %69.86 ± 0.25, %86.6 ± 2.55% ve %74.01 ± 2.32'ye yükseldiği belirlenmiştir.

Bu çalışma *Scenedesmus*, *Hindakia* ve *Micractinium* türlerinde yüksek miktarda fenolik bileşik olduğunu gösteren ilk çalışmadır. Ayrıca, ilk defa bu çalışmada, *Hindakia* ve *Micractinium* türlerinin antioksidan kapasitesi gösterilmiş ve iki farklı büyütme koşulunda elde edilen biyoaktif bileşikler kıyaslanmıştır.

Anahtar Kelimeler: Yeşil Mikroalgler, Oksidatif Stres, Antioksidan Kapasite, Biyoaktif Bileşikler, Hücre-koruyucu Aktivite

To my family

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

BBM: Bold's basal medium

DMSO: Dimethyl sulfoxide

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

DW: Dry weight

FITC: Fluorescein isothiocyanate

FRAP: Ferric reducing antioxidant power

GAE: Gallic acid equivalents

H_2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate

H_2O_2: Hydrogen peroxide

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBS: Phosphate-buffered saline

PI: Propidium iodide

PUFA: Polyunsaturated Fatty Acid

QE: Quercetin equivalents

ROS: Reactive oxygen species

RP-HPLC: Reversed phase high performance liquid chromatography

TAP: Tris Acetate Phosphate

TE: Trolox equivalents

CHAPTER 1

INTRODUCTION

Accumulation of reactive oxygen species (ROS) at high levels in cells as a result of normal cellular activities as well as exposure to oxidative stress is attributed to many chronic degenerative diseases and conditions in humans including cardiovascular diseases, cancer and aging-related conditions such as Alzheimer's disease (Christen, 2000; Waris and Ahsan, 2006; Li et al. 2007; Sugamura and Keaney, 2011). Several enzymes including superoxide dismutase, glutathione peroxidase and catalase are involved in the cell's endogenous defense system against free radicals; however, certain conditions such as cigarette smoking, exposure to environmental pollutants and UV radiation, and unhealthy eating habits may exert additional stress. In that situation, humans rely on exogenous sources of antioxidants for additional support. Many studies have shown that a diet rich in fruits and vegetables can circumvent the negative effects of oxidative damage (Prior, 2003; Holt et al. 2009). The antioxidants found in high amounts in certain plant species serve as the natural inhibitors of ROS (Masella et al. 2005; Pandey and Rizvi, 2009). Both natural and synthetic antioxidants are also widely used in the food industry as additives to prolong the shelf life of food products. There is an ongoing search for replacement of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) with their natural counterparts due to concerns about the potential toxic and carcinogenic effects of the former (Ito et al. 1986; Shebis et al. 2013).

Vitamins (particularly, vitamin C and vitamin E), carotenoids and phenolic compounds are the three major groups of antioxidants (Freile-Pelegrín and Robledo, 2013; Landete, 2013). Among these, phenolic compounds or phenols comprise a broad range of structural characteristics with diverse physiological effects

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(Balasundram et al. 2006). Anti-carcinogenic and anti-proliferative activities of phenols on breast, colon, prostate, and human leukemia tumor cell lines as well as on animal models of various cancer types have been documented (Dai and Mumper, 2010). Additionally, consumption of dietary supplements rich in phenolic compounds, particularly in early developmental stages has been associated with positive cognitive outcomes in patients with Down syndrome and related disorders (Vacca et al. 2016). Other effects of phenolics that have been reported up-to-date are antimicrobial, anti-inflammatory and anti-biofilm activities (Jagani et al. 2009; Zhang et al. 2011; Borrás-Linares et al. 2015). The mechanisms of antioxidant activity include inhibition of enzymes such as glutathione S-transferase, NADH oxidase and protein kinase C or chelation of trace metals (e.g. iron or copper) that are involved in ROS production, scavenging free radicals and up-regulating the antioxidant defense pathways (Pietta, 2000). The overall structure and certain structural components of flavonoids, a distinct class of phenolic compounds have a significant contribution to the efficiency of the antioxidant activity. Besides fruits and vegetables, flavonoids can also be found in many forms in medicinal plants, herbs and spices, nuts, tea and cereals (Pietta, 2000).

Although a vast number of studies has focused on the antioxidant potential of terrestrial plants (Brewer, 2011), microalgae are emerging as an alternative source of antioxidants. Several studies reported the phenolic content and antioxidant activity of microalgae (Li et al. 2007; Hajimahmoodi et al. 2010; Guedes et al. 2011; Goiris et al. 2012; Machu et al. 2015). The vast diversity of microalgae with the ability to produce different metabolites, the ease of cultivation with minimal land requirement, the feasibility of growth under different conditions including wastewater make microalgae attractive candidates in the quest for different antioxidant sources. Some species of microalgae, particularly *Chlorella* and *Spirulina* are commercially available as natural food supplements and are known as "super-foods" due to their rich phytochemical content and health benefits. Other microalgae (e.g. *Nannocloropsis* sp., *Tetraselmis* sp.) with high polyunsaturated fatty acid (PUFA)

composition have been explored as fish feed in aquaculture (Guedes and Malcata, 2012; Sørensen et al. 2017).

In present study, antioxidant capacities, total phenolic contents, total flavonoid contents and carotenoid contents of three novel thermo-tolerant freshwater green microalgal strains; *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 were evaluated and compared. *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 were grown under mixotrophic conditions; while *Micractinium* sp. ME05 was cultivated under two different cultivation methods; namely, mixotrophic versus heterotrophic cultivation. The microalgal biomass samples were extracted using different solvents of varying polarity. The antioxidant capacities of solvent extracts of microalgal biomass were measured by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assays. The statistical correlation of results obtained from two different assays were calculated. Additionally, the contribution of total phenolic content, flavonoid content and carotenoid content to the antioxidant capacities were also investigated by statistical analysis. Furthermore, the amounts of twelve different phenolic contents; namely gallic acid, benzoic acid, 4-hydroxy benzoic acid, vanillic acid, syringic acid, cinnamic acid, coumaric acid, caffeic acid, chlorogenic acid, rosmarinic acid, quercetin and rutin were identified in the microalgal extracts by Reverse Phased High Pressure Liquid Chromatography (RP-HPLC). Solvent extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 that showed the highest antioxidant capacity and total phenolic content were further tested for their cytotoxic effects on human breast adenocarcinoma cell line (MCF-7) and human cervical cancer cell line (HeLa) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Finally, inhibitory effects of the microalgal extracts on H_2O_2-induced intracellular ROS production and cytoprotective effects were evaluated in MFC-7 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Free radicals

Free radicals are defined as molecular species that are capable of independent existence and have one or more unpaired electrons in their outer shells. The presence of unpaired electrons results in some common features such as high reactivity and instability. As a result, these radicals are able to either extract electrons from molecules or donate electrons to other molecules thereby behaving as reductants or oxidants (Bahorun et al. 2006; Lobo et al. 2010). In spite of their high reactivity, free radicals have a very short half-life in biological systems, usually less than 10^{-6} s (Young and Woodside, 2001; Halliwell and Gutteridge, 2015). The theory of free radicals was proposed at the beginning of the twentieth century and the term "free radicals" was initially used to define certain intermediate compounds in organic and inorganic chemistry. However, it was discovered that these radicals might also have critical functions in biological systems and account for detrimental processes within the cell, especially in aging. (Gilbert, 1981). The hypothesis on the free-radical associated aging process pioneered various innovative studies that significantly contributed to the current understanding of free radicals including reactive oxygen species, reactive nitrogen species and non-radical reactive species, and to the discovery of their roles in the development of diseases. These metabolites are now considered to be major players in diverse cellular and biochemical processes (Pham-Huy et al. 2008).

2.2 Reactive oxygen species and reactive nitrogen species

Free radicals can be generated from various elements, radical species involving oxygen and nitrogen namely; reactive oxygen species (ROS) and reactive nitrogen species (RNS) gained much interest due to the oxidative stress related pathophysiological conditions. In general, ROS and RNS are classified into two main groups; radicals and non-radicals (Table 2.1 and 2.2). The radical group contains compounds such as superoxide anion radical ($O_2^{•-}$), hydroxyl radical (•OH), peroxyl radical ($ROO^{•-}$), alkoxyl radical ($RO^{•-}$), nitric oxide radical ($NO^{•}$), nitrogen dioxide ($NO_2^{•}$). These compounds are named radicals due to the presence of at least one unpaired electron in the shells around the atomic nucleus.

Table 2.1 Some examples of biologically important radical and non-radical reactive oxygen species (Phaniendra et al. 2015).

Name	Symbol	Half-life
Radicals		
Superoxide anion	$O_2^{•-}$	1×10^{-6} s
Hydroxyl	$^{•}OH$	1×10^{-9} s
Peroxyl	$ROO^{•-}$	1×10^{-2} s
Alkoxyl	$RO^{•-}$	1×10^{-6} s
Non-radicals		
Hydrogen peroxide	H_2O_2	stable
Organic peroxide	ROOH	stable
Singlet oxygen	$^1O_2$	1×10^{-6} s
Molecular oxygen	O_2	10^2 s
Ozone	O_3	s
Hypochlorous acid	HOCl	stable (min)
Hypobromous acid	HOBr	stable (min)

On the other hand, the group of non-radicals contains a large variety of compounds that are not free radicals themselves but can easily lead to formation of free radicals. These substances produced in living cells are hydrogen peroxide (H_2O_2), organic peroxides, hypochlorous acid (HOCl), ozone (O_3), peroxynitrite ($ONOO^-$) and nitrosyl cation (NO^+). ROS and RNS are produced by living organisms as a result of

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cellular metabolism and function in physiological processes at low concentrations. However, at high concentrations, they cause adverse effects in cells by damaging biomolecules such as nucleic acids, proteins and lipids (Metodiewa and Kośka, 1999; Phaniendra et al. 2015).

Table 2.2 Some examples of biologically important radical and non-radical reactive nitrogen species (Phaniendra et al. 2015).

Name	Symbol	Half-life
Radicals		
Nitric oxide	$NO^{\bullet}$	s
Nitrogen dioxide	$NO_2^{\bullet}$	s
Non-radicals		
Peroxynitrite	$ONOO^-$	1×10^{-3} s
Nitrosyl cation	NO^+	s
Nitroxyl anion	NO^-	s
Dinitrogen trioxide	N_2O_3	s
Dinitrogen tetraoxide	N_2O_4	s
Nitrous acid	HNO_2	s
Nitryl chloride	NO_2Cl	s
Peroxynitrous acid	$ONOOH$	fairly stable

2.3 Production of free radicals in biological systems

In biological systems, free radicals are derived either from normal cellular processes or from external sources.

2.3.1 External sources of free radicals

Free radicals in biological systems result from various types of exogenous sources including environmental pollution, alcohol, cigarette smoke, heavy metals such as cadmium, mercury, lead and iron, some drugs such as gentamicin, bleomycin and cyclosporine, industrial chemicals and solvents, cooking and radiation.

The most prevalent external source is cigarette smoke which contains different types of free radicals, organic compounds and oxidants such as superoxide and nitric oxide radicals. Further, inhalation of these compounds into the lungs also increases degree of the oxidation through accumulation of neutrophils and macrophages (Church and Pryor, 1985). Ozone exposure is another example of external free radical sources. Even in the case of short-term exposure, ozone exposure can lead to the release of inflammatory mediators such as myeloperoxidase (MOP) and eosinophil cationic proteins (Hiltermann et al. 1999).

Exposure of living organisms to ionizing and non-ionizing irradiation is another major contributor to formation of free radicals. Exposure of cells to γ-irradiation leads to the generation of a wide range of radical and non-radical species (e.g. aqueous electron, hydroxyl radical and hydrogen peroxide) through ionization of intracellular water. Even exposure to non-ionizing radiation such as ultraviolet (UV) irradiation can induce the formation of free radicals and manifest its deleterious effects in two ways. The first mechanism is the direct absorption of radiations by the cell and its components, which further results in the formation of an excited state of molecules. For instance, exposure to UV irradiation has been shown to cause the formation of 8-oxo-7-8-dihydro-2′-deoxyguanosine (8-oxo-dGuo), an oxidatively modified guanine that is considered to be a biomarker for oxidative DNA damage. The second mechanism is mediated by endogenous or exogenous photosensitizers. Exposure of cells to UV irradiation results in the excitation of photosensitizers, riboflavins, bilirubin and other porphyrins, subsequently leading to the formation of free radicals and oxidative damage (Jager et al. 2017).

Transition metals are also able to generate free radicals, and thereby cause cellular damage. Most of these metal ions contain unpaired electrons and convert stable molecules into reactive radicals. Among different transition metals, copper and iron are the most abundant metals and are present in relatively higher concentrations. The most important mechanisms of metal induced free radical generation is Fenton reaction and metal-mediated Haber-Weiss reaction. Superoxide anion and hydrogen peroxide molecules react with iron or copper ions through these mechanisms in order

to generate hydroxyl radicals. In addition to the Fenton and Haber-Weiss reactions, some metal ions are proposed to react directly with cellular molecules and result in production of free radicals such as thiols radicals.

In spite of the presence of various environmental and external sources, foods constitute the predominant source of free radicals. The extent of oxidation products significantly increases with the amount of food consumed by organisms. As such, organisms are exposed to a larger degree of oxidants such as peroxides, aldehydes, oxidized fatty acids and transition metals. Food debris that reaches the intestinal tract places an enormous oxidative pressure on the intestinal-tractmucosa (Burton and Jauniaux, 2011; Birben et al. 2012).

2.3.2   Endogenous sources of free radicals

Despite exposure of organisms to free radicals originating from external sources is extremely high, exposure to endogenous sources of free radicals is more important and extensive, since it is an unceasing and constant process during the life span of the organism.

The reduction of oxygen to water in the mitochondria for energy production occurs through the transfer of electrons from reduced nicotinamide adenine dinucleotide (NADH) or reduced flavin adenine dinucleotide (FADH$_2$) to oxygen by a series of electron carriers. The electron transfer along the enzymes of the respiratory chain is not achieved at full efficiency, and the electron leakage into intracellular environment, particularly from complex I and complex III, leads to the formation of several oxygen derivatives, particularly superoxide anion radical. Due to the extent and vitality of oxidative phosphorylation, mitochondria serve as the predominant organelle responsible for production of free radicals and many other events throughout the cell cycle. The massive production of mitochondrial free radicals is further increased under certain conditions including hyperoxia, aging and increased glucose amounts as in diabetes. Interestingly, hypoxic conditions have been shown

to elevate the formation of electrons since the reduced amounts of oxygen to act as the final electron acceptor for complex IV leads to the accumulation of electrons (Burton and Jauniaux, 2011).

In addition to mitochondria, free radicals, in particular ROS, are also formed by the electron leakage from the shorter electron transport chain within the endoplasmic reticulum (ER). Some of the post-translational modifications such as formation of disulfide bonds during protein folding is an oxidative process, and a significant amount of superoxide radical in biological systems is produced within the ER. In some cases such as cells with a high secretory output and ER stress when repetitive attempts for folding of misfolded proteins, the production of ROS is elevated as well (Burton and Jauniaux, 2011).

Enzymatic reactions constitute another endogenous source of free radicals produced under physiological processes. Some enzymes cause generation of ROS as a by-product of their activity. For example, hydrogen peroxide is produced within the cell by the action of xanthine oxidase which catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid in purine catabolism. Contrarily, there are certain enzymes designed to produce free radicals. For instance, nitric oxide synthase cataylzes the synthesis of nitric oxide, an important signaling molecule in biological systems, via oxidation of L-arginine (Kohen and Nyska, 2002).

Leukocytes including, neutrophils, basophils, eosinophils, monocytes and lymphocytes also contribute to the formation of endogenous free radicals by generating significant amounts of ROS as a result of their action in the immune response. Once these cells are stimulated, they increase the oxygen consumption approximately by 20-fold, which is followed by the increased glucose utilization and increased production of nicotinamide phosphate dinucleotide (NADPH) in a process referred to as the respiratory burst. NADPH acts as an electron donor for an enzymatic complex, named NADPH-oxidase, in the plasma membrane. NADPH oxidase complex catalyzes the production of superoxide radicals using electrons and oxygen. A dismutation reaction occurs with the superoxide radical in order to

produce hydrogen peroxide, which is followed by the formation of hydroxyl radical via the metal-mediated Fenton and/or Haber-Weiss reactions. Hypochlorous acid, on the other hand, is generated through the oxidation of chloride ions in the presence of hydrogen peroxide by the neutrophilic enzyme, myeloperoxidase (MOP) (Droge, 2002; Valko et al. 2007; Pacher et al. 2007).

Enzymatic free radical formation

$$\text{Hypoxanthine} + O_2 + H_2O \xrightarrow{\text{Xanthine oxidase}} \text{Xanthine} + H_2O_2$$

$$\text{NADPH} + 2O_2 \xrightarrow{\text{NADPH oxidase}} \text{NADP}^+ + 2O_2^{\bullet-} + H^+$$

$$\text{L-arginine} + O_2 + \text{NADPH} \xrightarrow{\text{Nitric oxide synthase}} NO^\bullet + \text{citrulline}$$

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{Xanthine oxidase}} \text{Uric acid} + H_2O_2$$

$$Cl^- + H_2O_2 + H^+ \xrightarrow{\text{Myeloperoxidase}} HOCl + H_2O$$

Non-enzymatic free radical formation

$$Fe^{2+} + H_2O_2 \longrightarrow {}^\bullet OH + OH^- + Fe^{3+} \qquad \text{Fenton reaction}$$
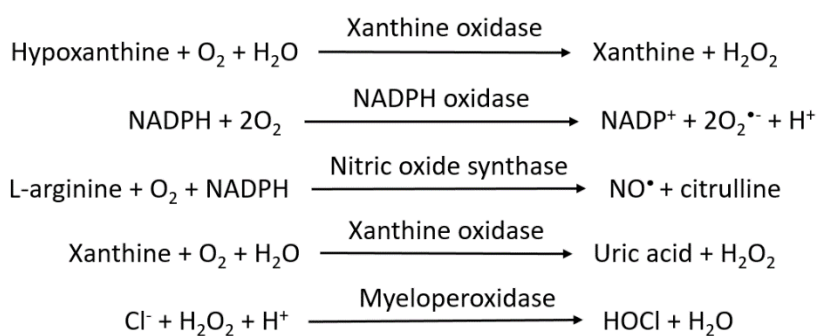
$$O_2^{\bullet-} + H_2O_2 \longrightarrow {}^\bullet OH + OH^- + O_2 \qquad \text{Haber-Weiss reaction}$$
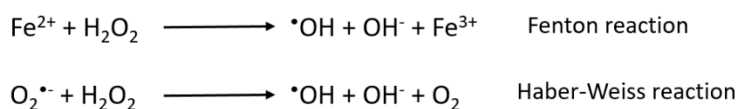
Figure 2.1 Examples of free radical reactions (Droge, 2002; Kohen and Nyska, 2002; Valko et al. 2007).

2.4 Oxidative stress

Although free radicals are necessary for many biological processes including signal transduction, maturation of cellular structures and defense against pathogens, accumulation of excess amounts of free radicals, whether they are endogenous or exogenous, results in oxidative damage. This phenomenon is called oxidative stress, a deleterious process that can seriously damage membrane structures, cellular functions and cellular components such as proteins, nucleic acids and lipids (Figure 2.2). Oxidative stress occurs when the balance between formation and neutralization of free radicals is impaired. This imbalance might arise from either depletion of

antioxidants or accumulation of oxidants. When living organisms are exposed to oxidative stress, cells attempt to neutralize the harmful effects of oxidants and reestablish the oxidation-reduction balance by activation or inactivation of genes coding for transcription factors, structural proteins and enzymes. In the case of insufficient counteraction, accumulation of free radicals within the cell leads to alterations in DNA structure, proteins and lipids, and results in activation of stress-induced transcription factors, and triggers production of pro-inflammatory and anti-inflammatory cytokines (Valko et al. 2007; Birben et al. 2012).
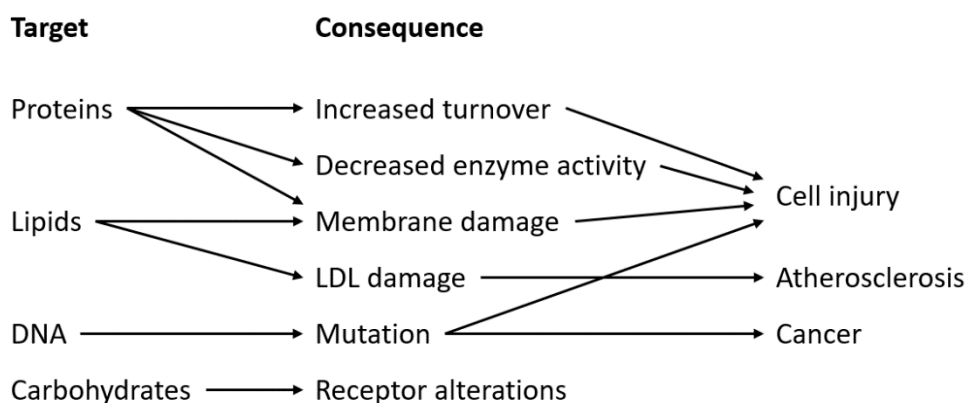
Figure 2.2 Effects of oxidative stress on various biomolecules and their consequences (Bagchi and Puri, 1998).

2.4.1 Effect of oxidative stress on lipids

Among various biomolecules, lipids, particularly membranes, are the most vulnerable cellular components to the oxidation due to the presence of high amounts of polyunsaturated fatty acids (PUFAs) in their structures. The oxidative damage to lipids, known as lipid peroxidation, can disorganize the lipid bilayer arrangement of membranes thus leading to inactivation of membrane-bound proteins, receptors and enzymes, and increased membrane permeability.

The lipid peroxidation occurs in three stages: initiation, propagation and chain termination (Figure 2.3). In the initiation stage, a reactive oxygen metabolite such as

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hydroxyl radical and hydroperoxyl radical abstracts a hydrogen atom from a methylene group in the lipid resulting in the formation of the carbon-centered lipid radical (L˙). Then, the lipid radical reacts with oxygen to form a lipid peroxy radical (LOO˙) in the propagation stage. The peroxyl radical is the carrier of the chain reaction and can further oxidize another lipid molecule generating a new lipid radical and a lipid hydroperoxide (LOOH). In the termination stage, one lipid peroxy radical react with another lipid peroxy radical or antioxidant forming a non-radical product. Since the lipid peroxidation is performed in a chain reaction, a single initiation reaction can result in complete peroxidation of the unsaturated lipids in the membrane. Antioxidant molecules that are able to counteract this process are described as chain-breaking antioxidants. Monounsaturated fatty acids (MUFAs) that have only one double bond, and saturated fatty acids that do not have any double bonds can undergo oxidation but not a chain lipid peroxidation reaction. Further, products of lipid peroxidation such as malondialdehyde (MDA) and unsaturated aldehydes can diffuse from the original site to another parts of the cells and inactivate cellular proteins by forming protein cross-linkages (Ayala et al. 2014).

$$LH + R^• \longrightarrow L^• + RH \qquad (i)$$

$$L^• + O_2 \longrightarrow LOO^• \qquad (ii)$$

$$LOO^• + LH \longrightarrow LOOH + L^• \qquad (iii)$$

$$LOOH \longrightarrow LO^• + LOO^• + aldehydes \qquad (iv)$$
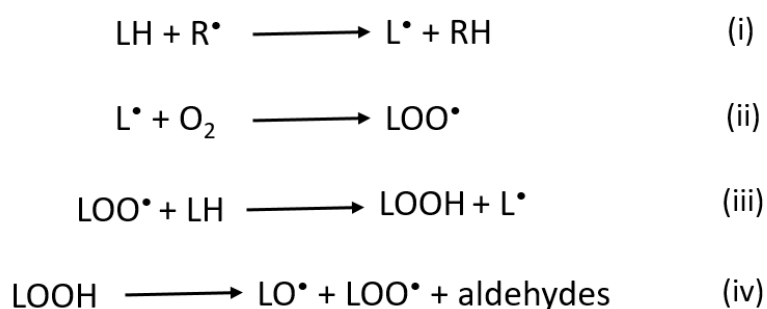
Figure 2.3 Reactions in the lipid peroxidation process; (i) initiation stage, (ii) and (iii) propagation stages, and (iii) termination stage (Ayala et al. 2014).

2.4.2 Effect of oxidative stress on proteins

Proteins, a major group of cellular constituents, can also be subject to deleterious effects of oxidative stress. Among different free radicals, hydroxyl and alkoxyl

radicals, and RNS are the prevalent causes of protein damage. Following the interaction with these radical compounds, proteins might undergo direct and indirect damage including peroxidation, fragmentation of the peptide chain, cross-linking, changes in the tertiary structure, alteration of the electrical charge, and oxidation of specific amino acids and thereby resulting in increased susceptibility to proteolysis by specific proteases. Cysteine and methionine residues within the peptide chains constitute the most susceptible sites to oxidation. Oxidation of thiol groups and methionine residues of proteins have been shown to induce conformational changes, loss of protein structure and protein degradation. Particularly, enzymes that contain metal ions on their active sites are more vulnerable to the metal catalyzed oxidation. The common outcome of protein damage by oxidative stress is the loss of activity, modified cellular functions, disruption of membrane potentials and alterations in the level of cellular proteins (Stadtman and Levine, 2003; Birben et al. 2012).

2.4.3   Effect of oxidative stress on DNA

Even though DNA is a well-protected molecule, free radicals are able to reach out to it and cause different types of damages such as single- and double-strand breaks, base or deoxyribose sugar modifications, mutations, and cross-linking with proteins. Most of these modifications on DNA are attributed to various types of pathophysiological conditions and disorders. Not all types of free radicals can cause DNA damage, but the most known example is hydroxyl radicals. Following exposure of DNA to hydroxyl radicals, it leads to the oxidative damage of sugar and/or heterocyclic moieties in oligonucleotides through two main mechanisms; addiction reaction and abstraction reaction. The former leads to the generation of OH-adduct radicals of DNA bases (Figure 2.4A), while the latter yields the allyl radical of thymine and carbon-centered sugar radicals (Figure 2.4B).

As illustrated in Figure 2.4A, hydroxyl radical reacts with the guanine at its C-8 position to produce the C-8-hydroxy-adduct radical of guanine, like those induced by UV irradiation. Then, C-8-hydroxy-adduct is converted to the 2,6-diamino-4-

hydroxy-5-formamidopyrimidine after reduction and opening reactions. However, the oxidation reaction results in the conversion of C-8-hydroxy-adduct radical of guanine to 8-hydroxyguanine, a biomarker for oxidative DNA damage. The hydroxyl radical also attacks the heterocyclic moiety of cytosine and thymine at C6- and C5-positions, resulting in the corresponding -OH adduct radicals. The oxidation of C5-OH and C6-OH adducts produces the unstable intermediate molecules, thymine glycol and cytosine glycol, respectively.
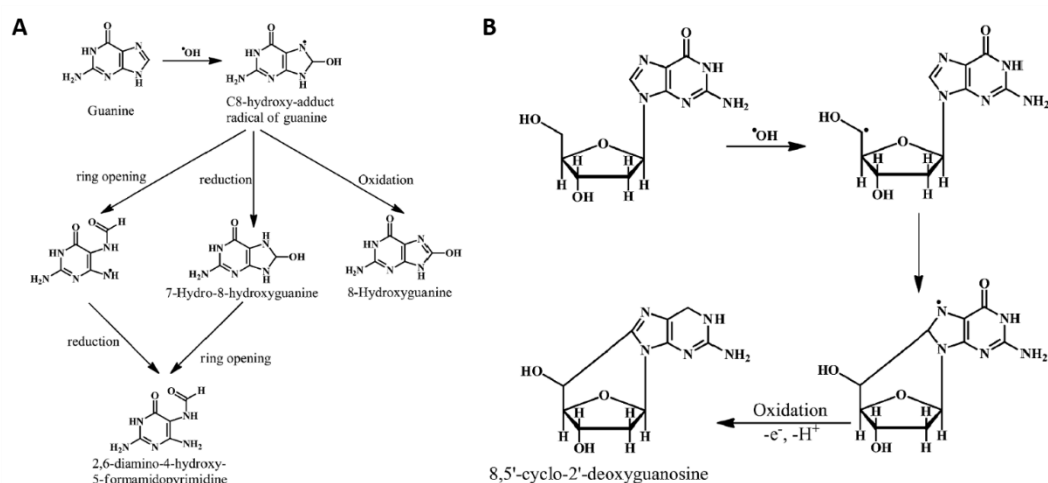
Figure 2.4 Reactions of hydroxyl radical with (A) the guanine and (B) the sugar moiety (Nimse and Pal, 2015).

Furthermore, the hydroxyl radical reacts with deoxyribose moiety of DNA by abstracting a hydrogen atom from C5′ position as shown in Figure 2.4B. Upon abstraction of the hydrogen atom, C5′-centered radical of the deoxyribose moiety is added to the C8 position of the purine ring in the same nucleoside by a an intramolecular cyclization reaction, giving rise to the generation of 8,5′-cyclopurine-2′-deoxynucleosides such as 8,5′-cyclo-2′- deoxyadenosine (cdA) and 8,5′-cyclo-2′- deoxyguanosine (cdG). This unusual puckering of the sugar moiety results in significant distortion in the DNA double helix and strand breaks (Jaruga and Dizdaroğlu, 2008; Nimse and Pal, 2015).

2.5   Antioxidants

The human body has various defense mechanisms to counteract the deleterious effects of oxidative stress, such as antioxidants, either formed *in vivo* or externally supplied through diet. Functions of the antioxidants are mainly neutralization of the excess of oxidants, protection of the cells and cellular components against the harmful effects of oxidants and assistance in the prevention of diseases (Pham-Huy et al. 2008). Antioxidants neutralize the oxidation by inhibition of free radical generation or by prevention of free radical propagation through different mechanisms: scavenging the species that trigger oxidation, chelating metal ions that are responsible for generation of free radicals, quenching super oxide anion, breaking oxidative chain reaction, and lowering localized oxygen levels (Brewer, 2011). Although antioxidants are classified in multiple ways such as based on their activity (e.g. enzymatic and non-enzymatic antioxidants), based on their solubility (e.g. water-soluble and lipid-soluble antioxidants) and based on their size (small-molecule and large-molecule antioxidants), the most common categorization is performed according to their sources; endogenous and exogenous antioxidants (Nimse and Pal, 2015).

2.5.1   Endogenous antioxidants

Endogenous (formed *in vivo*) antioxidants exist in either enzymatic or non-enzymatic forms. The major enzymatic antioxidants are superoxide dismutase, catalase, and glutathione peroxidase. Non-enzymatic antioxidants are produced by metabolism in the human body and comprise a wide range of molecules such as glutathione, metal-chelating proteins, melatonin, lipoid acid, coenzyme Q10 and L-arginine (Pham-Huy et al. 2008).

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2.5.1.1   Superoxide dismutase

One of the most effective intracellular antioxidant enzymes is superoxide dismutase (SOD), which catalyzes dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide (Table 2.3). In humans, three forms of SOD have been identified; cytosolic copper/zinc SOD (Cu/Zn-SOD), mitochondrial manganese SOD (Mn-SOD) and extracellular superoxide dismutase (EC-SOD). Cu/Zn-SOD is considered the first line of antioxidant defense, and specifically catalyzes the dismutation of the superoxide anion to oxygen and water. The respiratory system in mitochondria is primary source of reactive oxygen species, and Mn-SOD catalyzes the two step dismutation of superoxide anions through the cycling reaction of Mn(III) to Mn(II) and back to Mn(III). Therefore, it is considered one of the most efficient antioxidant enzymes in the body. EC-SOD is mainly found in the interstitial spaces of tissues and also in extracellular fluids. Therefore, EC-SOD is responsible for a considerable portion of the SOD activity in plasma, lymph and synovial fluid (Valko et al. 2006).

2.5.1.2   Catalase

Catalase (CAT) is a tetrameric enzyme composed of four identical subunits each with a molecular weight of 60 kDa. It is located in the peroxisome, and catalyzes the conversion of hydrogen peroxide to water and oxygen (Table 2.3). CAT is one of the most efficient enzymes, and cannot be saturated by hydrogen peroxide at any concentrations. In addition to its high efficiency, it has a very high turnover rate; one molecule of catalase can convert about six million molecules of hydrogen peroxide to water and oxygen each minute. Impaired activity of hydrogen peroxide detoxification in various cancer types has been associated with the reduced CAT activity (Mates, 2000).

2.5.1.3 Glutathione peroxidase

There are two forms of glutathione peroxidase; selenium-independent also known as glutathione-S-transferase and selenium-dependent glutathione peroxidase. Selenium-dependent glutathione peroxidases (GPx) are considered more essential in the protection against oxidants due to their function in the reduction of various hydroperoxides (e.g. ROOH and H_2O_2) using glutathione. There are four GPx isoenzymes found in humans, however all GPx enzymes are known to reduce peroxides by adding two electrons and forming selenoles (Se-OH). The action of GPx is accompanied by the tripeptide glutathione (GSH), which is found at high concentrations within cells. GPx catalyzes the decomposition of hydrogen peroxide or other peroxides into water or alcohol while simultaneously oxidizing GSH. Although the substrates of GPx and CAT are the same, GPx is capable of reacting with relatively lower concentrations of the substrate, thereby becoming the major defense mechanism against low levels of oxidative stress (Matés et al. 1999; Valko et al. 2006).

Table 2.3 Enzymatic antioxidants, their locations, substrates, and reactions (Nimse and Pal, 2015).

Enzyme	Location	Substrate	Reaction
Cu/Zn-SOD	Cytosol	$O_2^{\bullet-}$	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
Mn-SOD	Mitochondrial matrix	$O_2^{\bullet-}$	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
EC-SOD	Extracellular matrix	$O_2^{\bullet-}$	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
CAT	Peroxisome	H_2O_2	$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$
GPx	Various	H_2O_2	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$

2.5.1.4   Glutathione

The major endogenous non-enzymatic antioxidant is glutathione (GSH). It is considered to be the essential thiol-disulphide redox buffer of the cells, and found in high concentration in the cytosol (1-11 mM), nucleus (3-15 mM) and mitochondria (5-11 mM). While GSH is the reduced form, the oxidized form is named glutathione disulfide (GSSG) (Figure 2.5). The antioxidant capacity of GSH arises from the sulfur atom in thiol group, which is able to easily donate a single electron. For instance, GSH in the nucleus is mainly necessary for maintenance of the redox state of critical protein thiols (protein-SH). The oxidative stress causes the conversion of these proteins to either sulfenic acids (protein-SOH) or thiyl radicals (protein-S$^{\bullet}$). GSH reacts with these partially oxidized proteins and restores protein-thiols, which can be further oxidized irreversibly in the absence of GSH (Valko et al. 2006).
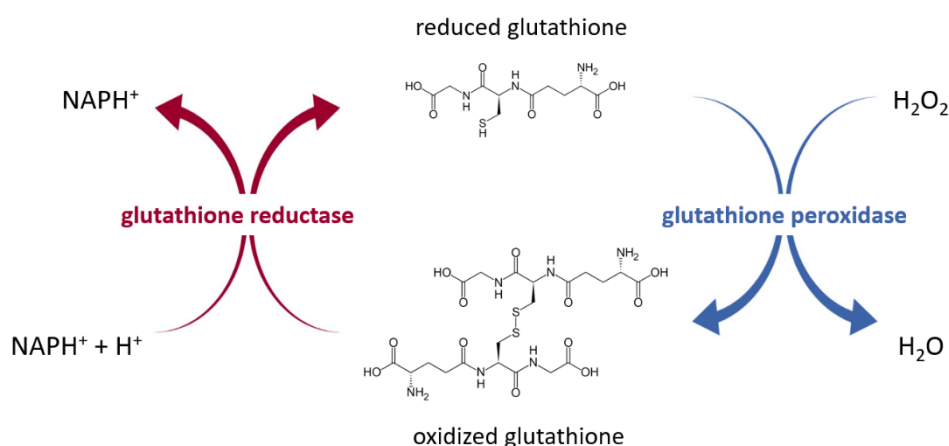
Figure 2.5 The reaction of glutathione catalyzed by glutathione reductase and glutathione peroxidase (Valko et al. 2006).

2.5.2   Exogenous antioxidants

Although the human body is equipped with endogenous antioxidant defense systems (either enzymatic or non-enzymatic), humans rely on exogenous sources of antioxidants for additional support. These antioxidant compounds comprise a wide

range of naturally occurring molecules such as phenolic compounds, flavonoids, carotenoids, vitamins E and C, and tocopherols. Besides, exogenous and endogenous antioxidants are known to co-operate synergistically in order to maintain or restore the redox homeostasis. Humans are not able to synthesize these compounds *de novo*, thus plant foods (fruits and vegetables) are the essential source of these natural antioxidants (Valko et al. 2006).

2.5.2.1   Vitamin E

Vitamin E is a fat-soluble vitamin with high antioxidant capacity and exists in eight different stereoisomers, namely; α, β, γ and δ tocopherols, and α, β, γ and δ tocotrienols (Figure 2.6). The most active form of Vitamin E in humans is α-tocopherol. Since it is fat-soluble, α-tocopherol is mainly found in the hydrophobic interior side of the cell membrane and constitutes the primary antioxidant defense system against lipid peroxidation by acting as a chain-breaker. It neutralizes lipid peroxyl radicals (LOO˙) by donating electrons and thereby ends the lipid peroxidation. Tocopheroxyl radicals formed as a result of this reaction are relatively stable and cannot sustain the lipid peroxidation process. Although Vitamin E serves as an effective antioxidant mechanism against lipid peroxidation by scavenging lipid peroxyl radicals, it is not efficient against other radicals such hydroxyl and alkoxyl radicals (Pham-Huy et al. 2008; Nimse and Pal, 2015).

Tocopherol	R_1	R_2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

Tocotrienol	R_1	R_2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H
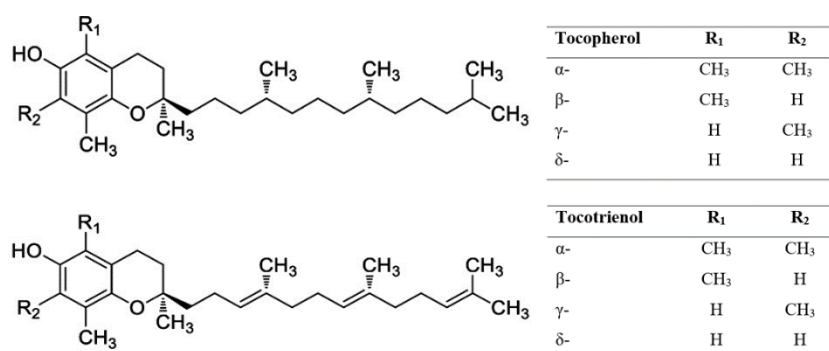
Figure 2.6 Basic chemical structures of Vitamin E stereoisomers (Xu et al. 2015).

2.5.2.2   Vitamin C

Vitamin C, also referred to as ascorbic acid, is a water-soluble vitamin. It primarily functions as a free radical scavenger in aqueous environments of the body and thereby acts as an antioxidant system. However, it is also involved in various biosynthetic pathways and particularly essential for synthesis of collagen, carnitine and neurotransmitters. Therefore, it is proposed to be associated with immune-modulatory, anti-atherogenic and anti-carcinogenic activities. Vitamin C works synergistically with carotenoids and antioxidant enzymes.

Vitamin C is a di-acid ($AscH_2$) due to the presence of two ionizable hydroxyl groups in its structure. However, it is mainly found in the ascorbate form ($AscH^-$) at physiological pH. Ascorbate reacts with radicals by donating an electron, and results in the formation of ascorbate free radical ($AscH^{\bullet}$). The final reaction of ascorbate oxidation is the conversion of ascorbate free radical to dehydroascorbate radical ($Asc^{\bullet -}$) which is a poorly reactive compound, and therefore considered to terminate the peroxidation chain reaction (Figure 2.7). Level of this radical is generally used as a biomarker for oxidative stress in biological systems (Kojo, 2004; Valko et al. 2006).
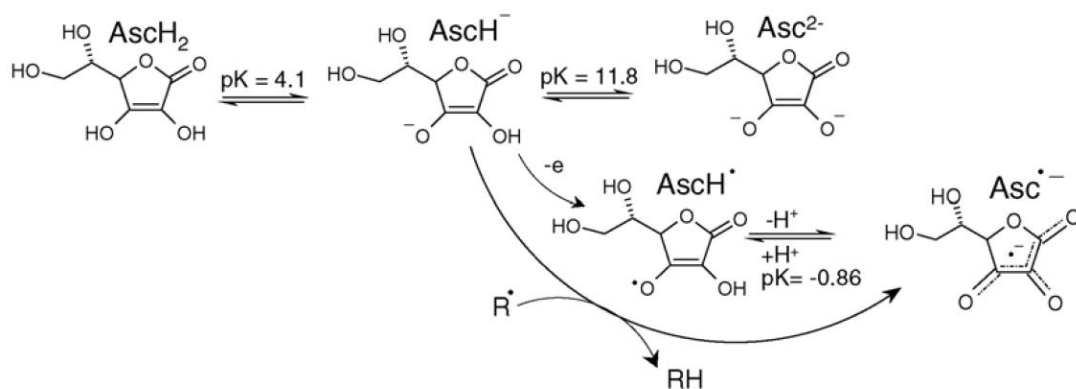
Figure 2.7 Mechanism of the reaction between ascorbic acid and free radicals (Valko et al. 2006).

2.5.2.3   Carotenoids

Carotenoids are naturally occurring pigments that are found in photosynthetic organisms such as plants, algae and cyanobacteria. In photosynthetic organisms, carotenoids have a wide range of vital functions including serving as light harvesting pigments for photosynthesis and being involved in the photosynthetic apparatus as basic structural units. There are more than 600 carotenoids, generally classified into two main groups: carotens (hydrocarbon carotenoids) and xanthophylls (oxygenated derivatives). Carotenoids are known to be effective scavengers against different types of ROS, particularly the peroxyl radicals. These radical molecules are produced as a result of lipid peroxidation, and can lead to the membrane damage and injury. Thus, scavenging of peroxyl radicals are important for the prevention of lipid damage and interruption of the chain reaction. Carotenoids are considered as a key factor in the protection of cellular membranes and their components such as lipoproteins due to their peroxyl radical scavenging activity (Lu and Li, 2008; Yahia et al. 2017).
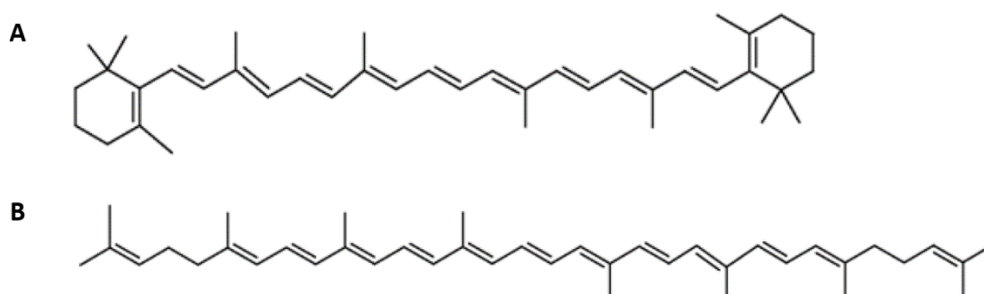
Figure 2.8 Basic chemical structures of (A) β-carotene and (B) lycopene (Valko et al. 2006).

The antioxidant activity of carotenoids originates primarily from their conjugated double-bonded structures which are able to delocalize unpaired electrons in free radicals. For instance, lycopene and β-carotene are the exemplary antioxidant carotenoids with high number of conjugated double bonds in the molecular structures (Figure 2.8). Therefore, these compounds are able to physically quench singlet

oxygen radicals without degradation and chemically scavenge various free radicals such as the hydroxyl, peroxyl and superoxide radicals (El-Agamey et al. 2004).

2.5.2.4   Phenolic compounds

Phenolic compounds represent one of the most widely occurring groups of secondary metabolites. These compounds are involved in various physiological processes such as growth and reproduction in plants and algae. Therefore, phenolic compounds have been shown to display various physiological properties including anti-allergenic, anti-microbial, anti-inflammatory and antioxidant activities. Structurally, phenolic compounds consist of a wide range of molecules, but generally characterized by an aromatic (phenolic) ring having one or more hydroxyl groups. Besides, phenolic compounds can be present as conjugates with simple or complex sugars and linked to other phenolic groups. Although this structural diversity causes different types of classification, phenolic compounds are basically categorized into two main groups based on the number of phenol rings present in their structure: phenolic acids and polyphenols (Sauceda et al. 2017).

2.5.2.4.1   Phenolic acids

Phenolic acids contain a single phenol ring bearing one carboxylic acid group and one or more hydroxyl groups. Based on the carbon units attached to the phenol ring, phenolic acids are divided into two subgroups, namely; hydroxybenzoic acids and hydroxycinnamic acids (Figure 2.9). Hydroxybenzoic acids have a general structure of C6-C1 and include gallic, vanillic, syringic and p-hydroxybenzoic acids. On the other hand, hydroxycinnamic acids, derived from cinnamic acid, are aromatic compounds with a three-carbon side chain, thus having the C6-C3 basic skeleton. The most common hydroxycinnamic acids are caffeic, *p*-coumaric, ferulic and sinapic acids. However, hydroxycinnamic acids can be also present as simple esters

with other molecules. The most common example is chlorogenic acid, an ester of caffeic acid and quinic acid (Goleniowski et al. 2013).

Hydroxybenzoic acids	R₁	R₂	R₃	R₄		Hydroxycinnamic acids	R₁	R₂	R₃	R₄	R₅
p-Hydroxybenzoic acid	- OH	-H	-H	-H		p-Coumaric acid	- OH	-H	-H	-H	-H
Vanillic acid	- OH	-H	-OCH₃	-H		Caffeic acid	- OH	-OH	-H	-H	-H
Gallic acid	- OH	-OH	-OH	-H		Sinapic acid	- OH	-OCH₃	-OCH₃	-H	-H
Syringic acid	- OH	-OCH₃	-OCH₃	-H		Ferulic acid	- OH	-OCH₃	-H	-H	-H
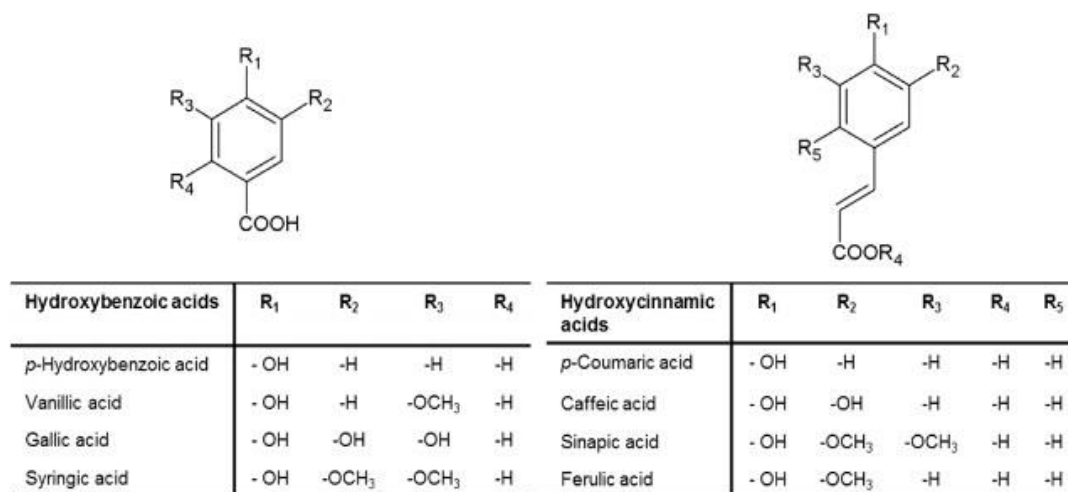
Figure 2.9 Basic chemical structures of hydroxybenzoic acids and hydroxycinnamic acids (de Córdova and Medina, 2014).

2.5.2.4.2 Polyphenols

Polyphenols are phenolic compounds that contain more than one phenol ring and divided into two main groups based on differences in their chemical structures; flavonoids and non-flavonoids.

The non-flavonoids group contains tannins, stilbenes and lignans. Tannins, the major group of non-flavonoids, are water-soluble polyphenols capable of forming cross-linkages with alkaloids, polysaccharides and proteins. Tannins may be further sub-divided into hydrolysable and condensed tannins. Hydrolysable tannins contain a central core of glucose or other polyhydric groups which are esterified either with gallic acid (gallotannins) or with hexahydroxydiphenic acid (ellagitannins). Condensed tannins, on the other hand, are polymers of polyhydroxyflavan-3-ol monomers linked through an interflavan carbon bond and structurally more complex than hydrolyzable tannins (Dai and Mumper, 2010). The second sub-group of non-flavonoids is stilbenes that are composed of two aromatic rings connected by an ethane bridge. Most stilbenes are synthesized in plants only in response to infection

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or injury and quite low in the human diet. The last sub-group of non-flavonoids are diphenolic compounds also referred to as lignans. These compounds are formed by dimerization of cinnamic acid residues and involved in plant defense against various diseases and pests (Pandey and Rizvi, 2009). However, stilbenes and lignans are not considered to be significant in the human diet.

Flavonoids are the main group of polyphenols, constituting over half of the naturally occurring phenolic compounds. Flavonoids are low molecular weight compounds, composed of 15 carbon atoms and arranged in a C6-C3-C6 configuration. Essentially, they contain two aromatic rings (A and B) joined by a 3-carbon bridge, generally in the form of a heterocyclic pyran ring (C) (Figure 2.10). Substitution patterns of rings A and B including hydroxylation, methylation, oxygenation and glycosylation differ in each class of flavonoids, thereby giving rise to the different compounds. Flavonoids are further divided into six classes, according to differences in the pyran ring: flavones, flavonols, isoflavones, flavanones, flavanols and anthocyanidins, of which flavones (e.g. apigenin, tangeretin and luteolin) and flavonols (e.g. quercetin, rutin and kaempfeerol) are the most predominant types and structurally diverse (Laure et al. 2019).
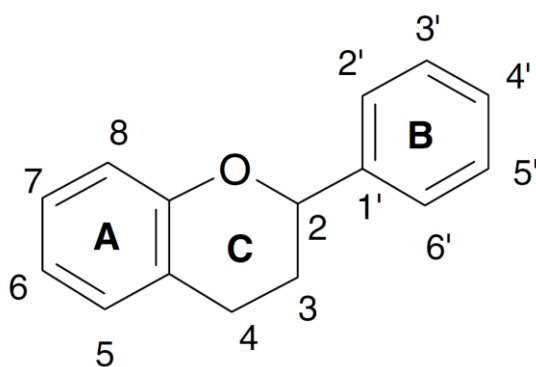
Figure 2.10 Basic chemical structure of flavonoids (Balasundram et al. 2006).

Phenolic compounds are potent antioxidant molecules due to their ability to chelate metal ions or to scavenge free radicals through donating hydrogen atoms or electrons. The antioxidant activity of phenolic compounds originates from their molecular structures, which is known as structure–activity relationships. This can be

seen in the case of phenolic acids in which the antioxidant capacity necessarily depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group. For instance, benzoic acid derivatives containing only one hydroxyl group at *ortho*- or *para*-position to the carboxylic acid do not exert antioxidant capacity, whereas the same is not true for 3-hydroxybenzoic acid. Further, it is known that the number of hydroxyl groups is a contributing factor to the antioxidant activity of phenolic acids, as seen in the case of trihydroxylated gallic acid. As compared to hydroxybenzoic acids, hydroxycinnamic acids show relatively higher antioxidant activity. The increased antioxidant capacity in hydroxycinnamic acids might be attributed to the presence of CH=CH-COOH group in their structures, which provides a higher degree of hydrogen donation and radical stabilization ability. The structure–activity relationship of polyphenols, particularly flavonoids, is usually more complex than that of phenolic acids as a result of the relatively more complex structures of polyphenols. The antioxidant activity of flavonoids are directly correlated with the structural features and types of substitutions on rings B and C. The key determinants of higher antioxidant activity in flavonoids include the followings: (i) the number and position hydroxyl groups in ring B, (ii) the presence of hydroxyl groups at 3´, 4´ and 5´ positions of ring B, (iii) a double bond between C-2 and C-3, conjugated with the 4-oxo group in ring C, (iv) a double bond between C-2 and C-3, combined with a 3-hydroxyl, in ring C, and (v) substitution of hydroxyl groups by methoxyl groups in ring B (Rice-Evans et al. 1997; Balasundram et al. 2006; Sauceda et al. 2017).

2.6   Applications of antioxidants

Natural antioxidant compounds have been shown to exert various protective biological properties including anti-inflammatory, anti-microbial, anti-proliferative, anti-mutagenic and anti-aging on the human health. Most of these beneficial properties are considered to result from the actions of dietary antioxidants (e.g. free radical scavenging and metal ion chelating) in the cell. Nevertheless, the mechanisms

through which natural antioxidant compounds display these activities are proposed to include their interaction with gene expression and signaling pathways, and related cellular machineries that mediate cell function under both normal and pathological conditions (Soobrattee et al. 2005; Kelsey et al. 2010).

Based on their beneficial biological properties, various types of antioxidants have attracted attention for their use as supplements, stabilizers and/or functional ingredients in food, pharmaceutical and cosmetic industries. Industrial products, in particular food products, are also vulnerable to oxidative damage which might occur upon exposure to air, heat and light. Essentially, deterioration and decomposition processes of those products are mainly related to oxidation reactions. Therefore, the incorporation of antioxidants is a major approach to maintain the overall quality of products by reducing or inhibiting oxidative damage. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butyl hydroquinone (TBHQ) and gallate derivatives (e.g. propyl, octyl and dodecyl) are commonly used as food supplements and additives in order to preserve the nutritional quality and to prolong shelf-life of food products. Although synthetic antioxidants have been widely preferred due to their high stability and low cost, concerns about safety issues have been raised over time. Several studies showed a direct correlation between the long-term intake of synthetic antioxidants and adverse effects including skin allergies, gastrointestinal complications and even carcinogenicity. In recent years, particular attention has been devoted to replacement of these antioxidants with natural counterparts (Kusumawati and Indrayanto, 2013; Lourenço et al. 2019).

To date, a large number of studies have focused on plants and plant based materials as natural sources of both potential and established antioxidants. Although available sources of antioxidants are diverse, the most common ones include agricultural and horticultural crops (e.g. cereals, fruits and vegetables), medicinal plants, spices and herbs (e.g. thyme, aloe vera, rosemary, sage, basil, cinnamon, ginger and turmeric). However, the production of plant based antioxidants might encounter a variety of difficulties such as insufficient amounts of antioxidants in intact plants, high production cost, limited availability and difficulty in cultivation. When taking these

issues into consideration, alternative sources that provide benefits such as simpler extraction routes, novel products not present in nature, more control over biosynthetic routes, independence from climatic factors and seasons, and shorter production cycles are in high demand (Matkowski, 2008). Microalgae are emerging as an alternative source for production of antioxidants and other bioactive compounds. The vast diversity of microalgae with the ability to produce different metabolites, the ease of cultivation with minimal land requirement, the feasibility of growth under different conditions including on wastewater make microalgae attractive candidates in the quest for different antioxidant sources (Bulut et al. 2009).

2.7   Microalgae

Algae are photosynthetic aquatic organisms. Based on their morphology and size, algae are typically classified into two major groups; macroalgae (multicellular) and microalgae (unicellular). Macroalgae, also referred to as seaweeds, are macroscopic organisms composed of multiple cells which are organized into structures similar to higher plants. In contrast, microalgae are microscopic single cells that are able to grow in various environments such as marines, freshwater (e.g. lakes, ponds and rivers) and groundwater (e.g. hot springs). Microalgae may be prokaryotic (cyanobacteria) or eukaryotic that are further sub-divided into different classes according to their pigmentation, life cycle and cellular structure. The most known groups are red algae (*Rhodophyta*), green algae (*Chlorophyta*), brown algae (*Phaeophyta*) and diatoms (*Bacillariophyta*).

Due to the ability of microalgae to adapt to diverse ecological habitats and various conditions such as extreme temperatures and pH, their biodiversity is enormous. In addition, their strong ability of adaptation in such conditions is associated with diverse features such as being rich in nutritional composition (e.g. starches, oils and proteins) and ability to accumulate important secondary metabolites (e.g. phenolic compounds and carotenoids). It is estimated that about 20,000-800,000 species exist of which only a small portion is identified, including several thousand algal species

described for research and only fifteen species used for manufacturing of industrial products (e.g. food, feed, drugs and certain chemicals). Therefore, there is a huge potential in the identification of novel algal species with economic benefits from natural environments (Chen et al. 2009; Suganya et al. 2016).

2.7.1   Biochemical composition of microalgae

Microalgal biomass is composed of various components; mainly carbohydrates, lipids and proteins, and pigments, vitamins and minerals. Many studies have been performed to investigate the nutritional content of microalgae. Table 2.4 shows the biochemical composition of selected microalgae species.

Table 2.4 Biochemical composition of selected microalgae expressed on a dry matter basis (% dry weight) (Lakmal et al. 2015; Suganya et al. 2016).

Microalgae	Proteins	Carbohydrates	Lipids
Anabaena cylindrical	43-56	25-30	4-7
Chlamydomonas rheinhardii	48	17	21
Chlorella ovalis	32	27	0.9
Chlorella vulgaris	51-58	12-17	14-22
Chlorella pyrenoidosa	57	26	2
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Porphyridium cruentum	28-39	40-57	9-14
Prymnesium parvum	28-45	25-33	22-39
Scenedesmus obliquus	50-56	10-17	12-14
Scenedesmus dimorphus	8-18	21-52	16-40
Spirulina maxima	60-71	13-16	6-7
Spirulina platensis	46-63	8-14	4-9
Tetraselmismaculata	52	15	3

2.7.1.1   Proteins

As shown in Table 2.4, microalgae have a high protein content, thereby considered to be an unconventional source of proteins. While the percentages vary among species, microalgae comprise of proteins up to 60% of their overall biomass. In addition, microalgae are able to synthesize all amino acids including the essential ones for humans and animals, and their amino acid composition compares favorably with that of other food proteins. For example, *Spirulina* is composed of about 60-70% of proteins depending on the strain, and it contains all of the essential amino acids. *Dunaliella* is another microalga that is cultivated at the industrial scale due to its high protein content. Proteins and peptides from marine microalgae have a great potential as functional ingredients of food products due to their certain features such as film and foaming capacity, gel forming ability and antimicrobial activity. In particular, phycobiliproteins that are a group of light harvesting proteins in algae have been shown to exert striking biological activities such as hepato-protective, anti-inflammatory, immune-modulating and antioxidant effects (Lordan et al. 2011; Lakmal et al. 2015).

2.7.1.2   Carbohydrates

Microalgae also contain high amounts of carbohydrates in the form of monosaccharides (e.g. glucose), structural and storage polysaccharides, and other saccharides. Total carbohydrate concentrations in microalgae generally range from 4% to 57% of dry weight (Table 2.4). There are various types of biologically important polysaccharides in microalgae, which are widely used as moisturizing and antioxidant ingredients in cosmetics and also as stabilizers, gelling agents and emulsifiers in food products.

In microalgae, polysaccharides greatly differ from one species to another. These biochemical and structural differences are also reflected in the enzymes involved in polysaccharide synthesis and modification. Most species of microalgae synthesize

heteropolymeric polysaccharides composed of mainly galactose, xylose and glucose in different proportions. Green microalgae synthesize starch in the form of two glucose polymers, amylopectin and amylose, whereas red microalgae produce a carbohydrate polymer known as floridean starch. However, *Chlorella vulgaris* is known to produce a homopolymer polysaccharide, β-1,3-glucan. Besides, polysaccharides obtained from *Chlorella* species are proposed to exert immune stimulatory and antimicrobial activities due to the presence of N-acetylglucosamide and N-acetylgalactosamine (Lordan et al. 2011; Chanda et al. 2019).

2.7.1.3   Lipids

The lipid content of microalgae varies between 1% and 40%, and under some conditions it can be 90% of dry weight. Generally, microalgal lipids are composed of glycerol and sugars esterified to saturated or unsaturated fatty acids (12 to 22 carbon atoms). Among all fatty acids, polyunsaturated fatty acids (PUFA) that contain more than one double bond in their structure are of particular interest since higher plants and animals lack the metabolic pathways to synthesize PUFAs of more than 18 carbons. In particular, PUFAs of omega-3 family (e.g. eicosapentaenoic acid and docosahexaenoic acid) and omega-6 family (e.g. linolenic acid and arachidonic acid) are essential in human diet due to their biological properties such as antioxidant and antimicrobial activities. Therefore, microalgae derived PUFAs have been commercially produced and used as nutritional supplements, food additives and pharmaceuticals. On the other hand, the most promising and common use of microalgal lipids is biodiesel production. Microalgae derived lipids are converted to biodiesel in the presence of a catalyst and an alcohol, and considered as an alternative and sustainable source of renewable energy (Spolaore et al. 2006; Michalak and Chojnacka, 2015).

2.7.1.4   Other compounds

In addition to the major components, microalgae also contain other types of compounds. Since marine microalgae are exposed to various kinds of environmental stress including rapid fluctuations of light intensity, oxygen, temperature and salinity, such exigent and aggressive environments result in the formation of stress related products including free radicals and oxidizing agents. Therefore, microalgae are able to synthesize potent molecules to counteract the stress and survive. Bioactive compounds produced by microalgae generally contain vitamins, carotenoids, phenolic compounds, sterols and growth factors.

One of the major components of microalgal cells is colored compounds known as pigments. They primarily participate in photosynthetic pathways of microalgae by absorbing and reflecting the visible light at certain wavelengths. The most common pigments are chlorophylls, carotenoids and phycobilins. The main carotenoids in microalgae are β-carotene, astaxanthin and lutein which are high-value compounds and can be used as food additives and supplements due to their high antioxidant properties. In particular, the nutritional relevance of β-carotene is attributed to its high pro-vitamin A activity which is essential for vision and proper functioning of the immune system. Besides, the action of β-carotene on prevention of cancer, aging, atherosclerosis and degenerative diseases is also suggested to be associated with its metal chelating and ROS quenching activities. On the other hand, astaxanthin is also linked to numerous health benefits such as antioxidant and immunomodulatory activities. Various strains of microalgae have been used as commercial production of carotenoids. *Dunaliella salina* is able to accumulate β-carotene up to 10% of its dry biomass, while astaxanthin is produced by *Haematococcus pluvialis* that may contain 4-5% astaxanthin per its dry biomass (de Jesus Raposo et al. 2013; Khan et al. 2018).

Phenolic compounds are considered to be one of the most abundant and important classes of natural antioxidants. Although phenolic compounds have been usually associated with plants, microalgae are also rich in these compounds. Phenolic

compounds are involved in important biological processes such as growth, reproduction, cell-wall formation and adhesion in algae. The variations in phenolic composition and content among algae originate from environmental, ecological and taxonomic differences. Especially, changes in environmental conditions such as light intensity, temperature and nutrient directly affect the composition of microalgae through their influence on photosynthetic processes. Although marine algae produce free radicals as a result of normal metabolic processes, stress conditions (e.g. UV exposure and increased light intensity) may lead to production of radicals at higher levels. In such a situation, microalgal cells may synthesize higher levels of phenolic compounds as a protective mechanism against stress factors (Freile-Pelegrín and Robledo, 2013).

The main types of phenolic compounds naturally produced in microalgae are phlorotannins, terpenoids, bromophenols and phenolic acids. Most of the studies on antioxidant properties of algae derived phenolic compounds have mainly focused on phlorotannins due to their biological properties such as protection against UV radiation, metal chelating and antibacterial activities. Depending on the habitat and environmental conditions, phlorotannins can constitute up to 25% of dry biomass. Among different species of algae, brown macroalgae and microalgae are considered to be the main sources of phlorotannins. In addition, bromophenols (e.g. 2-bromophenol, 4-bromophenol, 2,4-dibromophenol and 2,6-dibromophenol) and phenolic terpenoids have been detected in different species of algae and attributed to a variety of biological activities, including antioxidant, anticancer, antimicrobial and antiallergic properties. Despite the diversity of microalgae, identification and quantification of other types of phenolic compounds in other microalgal species is limited. However, recent studies have shown that different classes of phenolic compounds such as flavonoids (e.g. isoflavones, flavanones and flavonols) and phenolic acids (e.g. hydroxycinnamic acid and hydroxybenzoic acid) may also be found in microalgae. These findings indicated that microalgae are capable of producing a wide range of phenolic compounds, thus they can be evaluated as an

alternative source of natural antioxidants, particularly novel phenolic compounds (Safafar et al. 2015; Montero et al. 2018).

2.7.2　　　Cultivation of microalgae

Microalgal cultivation has received much attention in the last few decades due to their potential as a natural resource for biofuels as well as their ability to produce high-value chemicals and nutraceuticals. Therefore, the concept of "biorefinery" has been proposed for sustainable microalgal biomass production and sustainable processing of this biomass into a variety of commercial products and bioenergy. As depicted in Figure 2.11, the microalgal based biorefinery for biomass production include the following major steps: cultivation of microalgae, biomass harvesting, cell disruption, extraction and fractionation (Markou and Nerantzis, 2013).
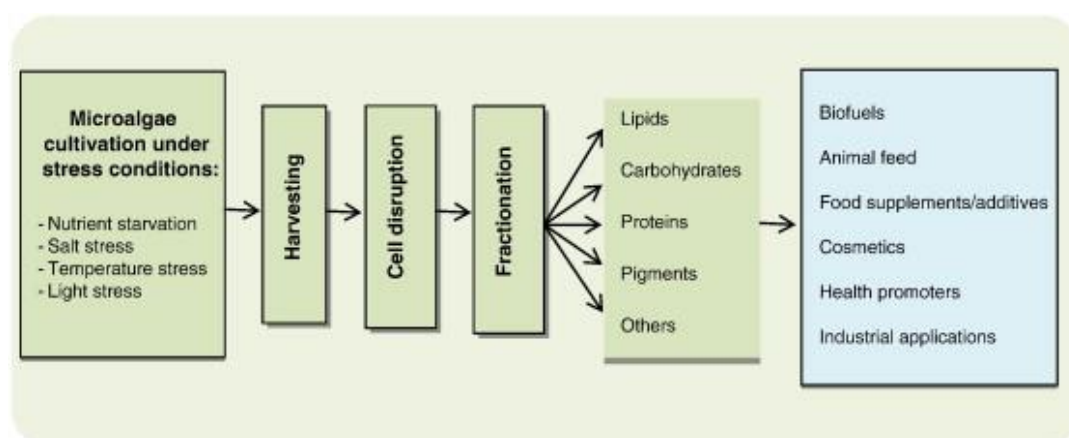
Figure 2.11 Major steps of microalgal based biorefinery concept (Markou and Nerantzis, 2013).

Despite the promising potential of microalgae as a sustainable and renewable resource, there are also problems in the biorefinery concept, such as cultivation of microalgae, industrial feasibility and availability. Cultivation of microalgae is considered to be the major challenge restricting the production capacity. Autotrophic, heterotrophic and mixotrophic cultivations are three main cultivation modes of microalgae. In autotrophic cultivation, microalgae grow and produce

organic substances through fixation of inorganic carbon, generally using CO_2 as carbon source and sunlight as energy source. In heterotrophic cultivation mode, microalgal growth depends on exogenous organic molecules as sources of carbon and energy. In mixotrophic cultivation, microalgae grow with both light and organic molecules as carbon and energy sources. In mixotrophic metabolism, microalgae perform photosynthesis using inorganic carbon, but the growth still relies on exogenous organic molecules as energy source.

2.7.2.1   Autotrophic cultivation

Autotrophic metabolism refers to reduction of CO_2 to carbohydrates. Since this metabolic pathway employs sunlight and CO_2, autotrophic cultivation is considered to be the simplest microalgal cultivation method. Besides, it is also economically feasible for large-scale production of microalgal biomass as compared to other methods. There are two main autotrophic cultivation systems, namely; open pond system and closed photo-bioreactor system. Technical practicability of each system mainly depends on properties of the microalgal strain used and climatic conditions.

Open pond production systems have been widely used for microalgae cultivation since 1950s. This system is further divided into two classes based on the source of water used in the system; natural waters (e.g. lakes, lagoons and ponds) and artificial ponds. Open pond system is suggested as a promising method for large-scale algal biomass production due to its simplicity and low-cost. However, it was reported that the cost of microalgal cultivation in open pond is still expensive for production of biofuel and other compounds. In addition, open pond system is susceptible to contamination by other algae and microorganisms, and the temperature difference between day and night is a major challenge that significantly affects the productivity and yield.

Closed photo-bioreactor system has been proposed to overcome the disadvantages of the open pond system. Although various types of closed photo-bioreactor systems

have been developed, plate photo-bioreactor tubular photo-bioreactor and vertical column photo-bioreactor constitute the three main types of closed photo-bioreactor systems. Among them, tubular photo-bioreactor has received more attention since it is more suitable for large-scale cultivation due to its relatively higher surface to volume ratio. Closed photo-bioreactor system provides more controlled environment for microalgal cultivation. Nevertheless, the costs of the closed systems considerably exceed the open pond systems.

Although autotrophic cultivation appears to be economically feasible, its drawbacks such as susceptibility to contamination and pollution, high costs of closed system and dependence on climatic conditions (e.g. light and temperature) complicate the autotrophic cultivation for large-scale microalgae cultivation for industrial purposes (Brennan and Owende, 2010; Zhan et al. 2017).
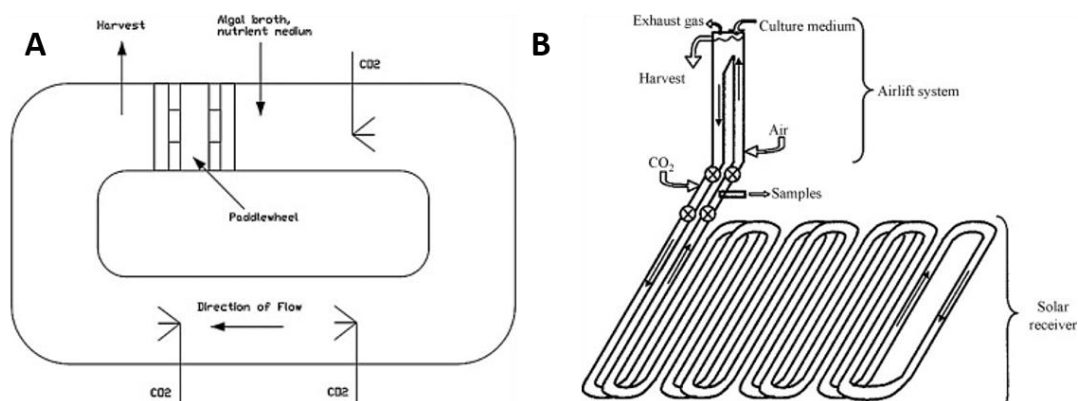
Figure 2.12 Basic plans of (a) open pond system and (b) horizontal tubular photo-bioreactor (Brennan and Owende, 2010).

2.7.2.2 Heterotrophic cultivation

Heterotrophic cultivation has also been widely preferred for large-scale production of microalgal biomass. In contrast to autotrophy, microalgae are cultivated in the presence of organic substrates (e.g. glucose) for energy and carbon acquisition in this process. Moreover, heterotrophic cultivation does not require light, which facilitates scale-up and large-scale production processes since cultivation can be performed in

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bioreactors or fermenters with smaller surface to volume ratio. Therefore, this system provides a higher degree of control over growth parameters and also yields higher cell densities and biomass. However, heterotrophic growth is not applicable to all microalgal species and has been observed in certain species of microalgae, which is a limiting factor for large-scale production potential of heterotrophic cultivation.

The most common carbon source in heterotrophic cultivation is glucose, which is a major concern due to its high cost. The cost inefficiency of heterotrophic production using glucose has led to search for alternative carbon sources. In this respect, various sources such as cellulose hydrolysis products, acetate and glycerol have been used for heterotrophic cultivation of microalgae. However, the type of organic carbon significantly affects the microalgal growth and biomass yield. Furthermore, numerous surplus materials in industrial wastes and sewage can also be reused in cultivation of microalgae in order to reduce the production cost. Therefore, novel approaches combining microalgae cultivation with waste products and even wastewater have been developed, and several microalgae species have been heterotrophically cultivated in carbon-rich waste products or in wastewater with additional organic nutrients. Engin et al. (2018) reported the heterotrophic cultivation of a green microalga, *Micractinium*, using different by-products of sugar refinery including molasses and vinasse as the nutrient source for biomass and lipid production (Engin et al. 2018a; Engin et al. 2018b; Engin et al. 2018c).

Main disadvantages of heterotrophic production of microalgal biomass are high costs due to organic carbon requirement, need for sterile growth media, susceptibility to bacterial and fungal contaminations, adverse effects of the contamination on culture vitality, reduced production of pigments and high-value phytochemicals due to cultivation under dark conditions, and production of CO_2 during growth. On the other hand, heterotrophic culture may provide some benefits such as accelerated growth rate, increased biomass and particularly lipid accumulation. Besides, it can be integrated with wastewater treatment and/or by-product processing in order to reduce the cost (Lowrey et al. 2015; Zhan et al. 2017).

2.7.2.3   Mixotrophic cultivation

Mixotrophic cultivation is defined as a combination of autotrophic metabolism and heterotrophic metabolism. In mixotrophic metabolism, microalgae are capable of using either metabolic process (autotrophic or heterotrophic) by performing photosynthesis as well as using organic carbon sources. Therefore, cell growth does not strictly depend on photosynthesis, and either light energy or organic materials may support the growth. Since cell growth is mainly influenced by the organic carbon supplement during both light and dark phases, light energy is not completely the limiting factor.

Mixotrophic cultivation combines the advantages of autotrophic and heterotrophic cultivation modes and partially or fully overcomes the disadvantages of both systems. Thus, increased growth rates and reduced photo-inhibition have been observed in mixotrophic cultivation of microalgae. In addition, mixotrophic growth allows the production of photosynthetic and heterotrophic compounds during the dark-light cycle. This significantly decreases the influence of biomass loss during the dark phase and reduces the amount of organic sources used in the growth.

There are some limitations in the mixotrophic cultivation microalgae, such as (i) the requirement of light, organic carbon, carbon dioxide and oxygen, (ii) relatively less energy conversion in comparison to heterotrophic metabolism, and (iii) release of carbon dioxide. Nevertheless, aforementioned properties make the mixotrophic cultivation an attractive mode for the production of microalgae based biofuels and bioactive compounds (Brennan and Owende, 2010; Zhan et al. 2017).

The comparison and overall differences of these three cultivation modes are summarized in Table 2.5.

Table 2.5 Comparison of autotrophic, heterotrophic and mixotrophic cultivation modes (Zhan et al. 2017).

Cultivation mode	Carbon source	Energy supply	Advantage	Disadvantage
Autotrophic	CO_2	Light	(1) Low cost (2) High production of pigmentation and phytochemicals	(1) Low growth rate, low biomass and lipid accumulation (2) Need special bioreactors or high dependence of weather condition
Heterotrophic	Organic carbon source (e.g. glucose, glycerol, acetate)	Organic carbon source	(1) Higher growth rate, higher biomass and lipid accumulation (2) Bioreactors design with little constraints	(1) Higher cost (2) Need for sterile media and easy to be contaminated (3) Production of CO_2 (4) Dark conditions diminish the pigmentation and production of phytochemicals
Mixotrophic	CO_2 and organic carbon source	Light and organic carbon source	(1) Higher growth rate, higher biomass and lipid accumulation (2) Sustain of pigmentation and phytochemicals production (3) Decreased production of CO_2	(1) Higher cost (2) Need for sterile media and easy to be contaminated (3) Reduced energy conversion efficiency

2.7.3   Industrial applications of microalgae

As previously mentioned, microalgae represent an extremely diverse group of organisms with the ability to produce a wide range of biologically important compounds. These bioactive compounds have been used in various applications such as human and animal nutrition, cosmetics, pharmaceuticals, health and biofuels. Moreover, microalgae have also been used in carbon dioxide capture and wastewater treatment. Microalgae derived bioactive compounds are proposed as a green alternative to present sources of those compound, and offer environmental and economic benefits. Although the use of microalgae as an efficient and affordable energy resource is not predicted to happen in the short-term, microalgae based biotechnology is expanding and developing in the direction of a high value market (Olaizola and Grewe, 2019).

Large-scale production of microalgae for commercial purposes was initiated in the early 1960s in Japan with the culture of *Chlorella*. After a decade, other species of microalgae such as *Arthrospira* has been commercially cultivated, and the first use of algae in aquaculture appeared. By 1980, there were about 50 large-scale facilities mainly in Asia producing more than one ton of microalgae, which was followed by commercial production of *Dunaliella salina* in Australia as a source of β-carotene. Together with these, microalgal biotechnology has significantly grown and diversified for production of other high-value compounds. Today, it is estimated that microalgal market produces approximately 5000 tons of biomass and generates a turnover of about US$ 1.25×10^9 each year (Khatoon and Pal, 2015).

2.7.3.1   Human nutrition

The microalgal market for human nutrition is dominated by a few species, mainly due to their high protein content and nutritional value. The microalgal biomass is marketed in different forms including tablets, capsules and liquids. In addition, they can also be incorporated into various food matrices such as pasta, snack foods, gums

and beverages. Owing to the presence of various bioactive compounds with diverse chemical properties, they can function as both nutritional supplements and natural food colorants. The commercial applications are dominated by five main species of algae; *Arthrospira*, *Chlorella*, *D. salina* and *Aphanizomenon flosaquae* (Table 2.6).

Arthrospira represents the best known example of microalgae based dietary supplement, generally made from *Arthrospira platensis* and *Arthrospira maxima*, and also known as *Spirulina*. In addition to its high protein content and superior nutritional value, *Arthrospira* has been shown to be associated with health promoting effects such as mitigation of hyperlipidemia, suppression of hypertension, promotion of intestinal *Lactobacillus* growth, protection against renal failure and regulation of serum glucose level. A significant portion of *Arthrospira* biomass is produced in China and India. The largest producer in the world is Hainan Simai Enterprising located in China, which accounts for 25% of the total national output and almost 10% of the world output. Their *Spirulina* products are distributed in over 20 countries in the form of tablets and powder. *Chlorella* is another commercially produced green microalga. It is produced by over 70 companies worldwide, and Taiwan *Chlorella* Manufacturing and Co. is the largest supplier with 400 tons of dried biomass produced annually. The commercial importance of *Chlorella* arises from the presence of high amounts of β-1,3-glucan in its biomass, which is a potent immuno-stimulator, a free radical scavenger and a reducer of blood lipids. In addition to its health-promoting effects, *Chlorella* biomass is also used as coloring, taste and flavor adjusting agent in food products. *D. salina* is produced and used for its high β-carotene amount which can reach 14-15% of dry weight biomass. Various producers commercially offer *D. salina* powder as an ingredient of dietary supplements and functional foods. *A. flosaquae* is the last major commercial microalgal strain that has gained attention due to its high nutritional content. Its biomass contains high-value bioactive compounds such as B-complex vitamins, essential fatty acids and phycocyanin, therefore used alone or in combination with other supplements and nutraceuticals (Spolaore et al. 2006).

Table 2.6 Major microalgae commercialized for human nutrition (Khatoon and Pal, 2015).

Microalga	Major producers	Products	Production (t/year)
Arthrospira (*Spirulina*)	Hainan Simai Pharmacy Co. (China)	Powders, extracts	3000
	Earthrise Nutritionals (California, USA)	Tablets, powders, extracts	
	Cyanotech Corp. (Hawaii, USA)	Tablets, powders, beverages, extracts	
	Myanmar *Spirulina* factory (Myanmar)	Tablets, chips, pasta and liquid extract	
Chlorella	Taiwan *Chlorella* Manufacturing Co. (Taiwan)	Tablets, powders, nectar, noodles	2000
	Klötze (Germany)	Powders	
Dunaliella salina	Cognis Nutrition and Health (Australia)	Powders	1200
		β-carotene	
Aphanizomenon flosaquae	Blue Green Foods (USA)	Capsules, crystals	500
	Vision (USA)	Powder, capsules, crystals	

2.7.3.2 Animal nutrition

Another commercial use of microalgae is the incorporation its biomass into feed products for different types of animals ranging from fish (aquaculture) to pets and farm animals. Roughly, one third of the commercially produced algal biomass is used in animal feed products, and about 50% of the algal biomass used as feed supplement is obtained from *Arthrospira*. As compared to conventional feed preparations, microalgal biomass incorporated feeds has a higher nutritional content, therefore improve the physiology of animals. The main positive effects of microalgal biomass

is the improved immune response, resulting in growth promotion, resistance to diseases, antimicrobial action, improved gut function, stimulation of probiotic colonization, as well as improved feed conversion, reproductive performance and weight control.

In addition to *Arthrospira*, other species such as *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira* are frequently used in animal feeds. In order to be applied as feed supplement, a microalgal species has to meet various criteria: (i) firstly, it should be nontoxic, (ii) it should be of the correct size and shape for proper digestion and bioavailability, and (iii) it should also have a high nutritional value. The content of protein and polyunsaturated fatty acids such as eicosapentaenoic acid, arachidonic acid and docosahexaenoic acid, as well as vitamins is the major factor in terms of the nutritional value. For instance, some fatty acids are essential in aquaculture applications for the growth and metamorphosis of many larvae (Spolaore et al. 2006; Han et al. 2019).

2.7.3.3   Cosmetics and pharmaceuticals

Microalgal extracts that contain high-value bioactive compounds have been generally applied in face and skin care product formulations such as anti-aging creams, moisturizing and refreshing creams and emollients, and as an anti-irritant in peeling products. Another wide spread application is the inclusion of algal extracts in sun protection and hair care products. The most known examples are the incorporation of *Arthrospira* and *Chlorella* biomass into skin care products. For example, a protein-rich extract of *Arthrospira* has been claimed to repair early skin aging, tighten the skin and prevent the formation of stria. In addition, an extract of *Chlorella vulgaris* has been shown to promote the collagen synthesis in skin, thus stimulating tissue regeneration and acting as anti-wrinkle agent. Microalgae derived novel products are still under development. Recently, a bioactive ingredient from *Nannochloropsis oculata* with superior skin-tightening properties and another

ingredient from *D. salina* with stimulatory effect on cell proliferation and turnover have been also announced by cosmetic companies.

Polyunsaturated fatty acids (PUFAs) contain more than one double bond in their backbone with more than 18 carbons. Since higher plants and animals are unable to synthesize PUFAs, they are considered essential and obtained through diet. Many PUFAs function in the development of brain and nervous system. Particularly, docosahexaenoic acid (DHA) participates as a structural component in the grey matter of the brain, in the retina of the eye and in the heart tissue. It is found in a limited number of food products. Despite, fish oil is a rich source of DHA, concerns have raised due to accumulation of toxins in fish oil. Therefore, microalgae derived PUFAs such as DHA have been commercially manufactured in the form of various pharmaceutical products (Table 2.7) (Spolaore et al. 2006; Khatoon and Pal, 2015).

Table 2.7 Biologically important microalgal PUFAs (Spolaore et al. 2006).

PUFA	Potential application	Microorganism producer
γ-Linolenic acid	Infant formulas for full-term infants Nutritional supplements	*Arthrospira*
Arachidonic acid	Infant formulas for full-term/preterm infants Nutritional supplements	*Porphyridium*
Eicosapentaenoic acid	Nutritional supplements Aquaculture	*Nannochloropsis*, *Phaeodactylum*, *Nitzschia*
Docosahexaenoic acid	Infant formulas for full-term/preterm infants Nutritional supplements Aquaculture	*Crypthecodinium*, *Schizochytrium*

2.7.3.4   Bio-fertilizers

Although fertilizers significantly increase crop productivity, safety issues have raised due to potential effects of conventional fertilizers such as damage in soil texture and other environmental problems. These concerns have led to the development of bio-fertilizers which contain microorganisms. They are suggested to promote adequate supply of nutrients to the host, ensure their proper growth and development and protect the host from soil-borne diseases. Thereof, bio-fertilizers present an economical and eco-friendly alternative to chemical fertilizers. Microalgae have gained much attention as a rich source of bioactive compounds that can be used as bio-fertilizers.

In this context, nitrogen fixing blue-green algae have been used for the efficient production of rice. Beneficial effects of algae on crops have been reported not only for rice but also for other crops such as wheat, maize, oat, sugarcane, bean, cotton, tomato and lettuce. The beneficial effects of algae on crops originate from the enhanced atmospheric nitrogen fixing, consequently promoting the crop growth and yield. Additionally, algae produce complex organic carbon compounds that improve the structure, permeability and water-holding capacity of soil. The ability of biosynthesis of growth-promoting substances such as auxins, amino acids, sugars and vitamins at high levels also significantly contributes to the crop growth (Kaushik, 2007; Karthikeyan et al. 2007; Khatoon and Pal, 2015).

2.7.3.5   Biofuels

Because of increasing population and energy demand over the world, it is necessary to find novel renewable resources for energy production. Algae, particularly unicellular green microalgae, have been proposed as a potential renewable resource for the production of bioenergy and biofuels. The main advantages which make microalgae a potential new generation of feedstock for biofuel production are high oil content, rapid biomass production and high photosynthetic efficiency. In this

context, the use of microalgae as biofuel and biodiesel feedstock has gained interest from both researchers and entrepreneurs. Microalgae derived feedstock can be used directly or processed into fuels through biochemical or thermochemical conversion processes. Another major advantage of microalgae as a bioenergy and biofuel resource is that the cultivation of microalgae can be achieved with less freshwater input and less land requirement unlike plant based bioenergy and biofuel crops, therefore making the process sustainable and renewable (Markou and Nerantzis, 2013).

The increase in oil and fuel prices raised the bioenergy and biofuel production. Microalgal strains with high oil content can be produced in large-scale under optimized growth conditions and have the potential to yield 5,000-15,000 gal of oil per acre per year. The most significant distinctive feature of microalga based oils and biofuels is the yield which is over 200 times higher as compared to best performing plant oils. Despite the high productivity of microalgae based biodiesel, it cannot yet compete with petroleum fuel due to higher costs. However, it is estimated that decrease in petroleum sources will make microalgae biodiesel essential (Contreras et al. 2015; Khatoon and Pal, 2015).

2.8   Microalgal strains used in this study

In this study, three novel green microalgal species were used; *Scenedesmus* sp. ME02, *Micractinium* sp. ME05 and *Hindakia tetrachotoma* ME03. These green microalgal strains were previously isolated from thermal springs of Haymana, Ankara (latitude 39.4° N, longitude 32.48° E). Detailed morphological, physiological, biochemical and molecular characterization of the microalgal species was formerly performed by Onay et al. (2014). All three species exhibit thermo-tolerance and are able to adapt to a wide range of temperature for growth.

Scenedesmus sp. ME02 can withstand a wide temperature range between 10-50°C. It is composed approximately of 56% proteins and the total lipid content varies between 10-20% depending on different culture conditions and extraction methods

(Onay et al. 2016). The fatty acid composition is also highly variable and the polyunsaturated fatty acid (PUFA) content can be as high as 75% of total fatty acids when grown at 16 °C.

Hindakia tetrachotoma ME03 can be cultivated at temperatures between 25-50°C. Despite its slower specific growth rate, *Hindakia tetrachotoma* ME03 shows the highest biomass accumulation. The total lipid content of *Hindakia tetrachotoma* ME03 is around 8.7%, which is the lowest among other microalgal species. However, eicosenoic acid constitutes 58% of the lipid content, which is a monounsaturated omega-9 fatty acid and generally attributed to antioxidant and anti-inflammatory activities (Onay et al. 2014; Onay et al. 2016; Sonmez et al. 2016).

Micractinium sp. ME05 can be cultivated in a wide temperature range between 16-50°C, however the highest growth rate is obtained at 25°C. Its total lipid content is about 21.9% of the total dry weight under optimum conditions. The main fatty acids are linoleic acid, palmitic acid, and linolenic acid comprising 32.3%, 27.0% and 26.7% of total fatty acids, respectively. Moreover, this strain is able to grow under heterotrophic conditions using industrial waste products, molasses and vinasse as carbon sources for the large-scale and low-cost cultivation (Engin et al. 2018a; Engin et al. 2018b; Engin et al. 2018c).

2.9   Aim of the study

The aim of present study is to evaluate and compare antioxidant capacities, total phenolic contents, total flavonoid contents and carotenoid contents of three novel thermo-tolerant freshwater green microalgal strains; *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05.

Scenedesmus sp. ME02 and *Hindakia tetrachotoma* ME03 were grown under mixotrophic conditions; while *Micractinium* sp. ME05 was cultivated under two different cultivation methods, namely; mixotrophic versus heterotrophic cultivation.

The microalgal biomass samples were extracted using different solvents of varying polarity. The antioxidant capacities of solvent extracts of microalgal biomass were measured by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assays. The statistical correlation of results obtained from two different assays was calculated. Additionally, the contribution of total phenolic content, flavonoid content and carotenoid content to the antioxidant capacities was also investigated by statistical analysis.

Furthermore, the amounts of twelve different phenolic contents, namely; gallic acid, benzoic acid, 4-hydroxy benzoic acid, vanillic acid, syringic acid, cinnamic acid, coumaric acid, caffeic acid, chlorogenic acid, rosmarinic acid, quercetin and rutin were identified in the microalgal extracts by Reverse Phased High Pressure Liquid Chromatography (RP-HPLC).

Solvent extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 that showed the highest antioxidant capacity and total phenolic content were further tested for their cytotoxic effects against human breast adenocarcinoma cell line (MCF-7) and human cervical cancer cell line (HeLa) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Finally, inhibitory effects of the microalgal extracts on H_2O_2-induced intracellular ROS production and their cytoprotective effects were evaluated in MFC-7 cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), iron(III) chloride hexahydrate, , Folin-Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), hydrochloric acid (HCl), phosphate-buffered saline (PBS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hydrogen peroxide (H_2O_2), trolox, gallic acid, quercetin and the authentic phenolic compound standards used in chromatographic analysis were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), sodium acetate trihydrate ($CH_3COONa·3H_2O$) and sodium nitrite ($NaNO_2$) were supplied by AppliChem GmbH (Darmstadt, Germany). Solvents including dimethyl sulfoxide (DMSO), methanol, ethanol, ethyl acetate, acetone and hexane were purchased from Merck Company (Darmstadt, Germany). 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), propidium iodide (PI), trypan blue solution, trypsin-EDTA solution, growth media and medium supplements used in the cell culture were obtained from Thermo Fischer Scientific (Waltham, MA, USA). All chemicals and reagents used in the study were analytical or chromatography grade.

3.2 Algal strains

In this study, three green microalgal species, namely; *Scenedesmus* sp. ME02, *Micractinium* sp. ME05 and *Hindakia tetrachotoma* ME03 were used. These green microalgal strains were previously isolated from thermal springs of Haymana,

Ankara (latitude 39.4° N, longitude 32.48° E). Detailed morphological, biochemical, physiological and molecular characterization of the microalgal species was formerly performed by Onay et al. (2014). All three species exhibit thermo-tolerance and are able to adapt to a wide range of temperature for growth.

3.3   Culture and growth conditions

Cultures of the microalgal species were maintained mixotrophically in Tris-Acetate-Phosphate (TAP) growth medium either in petri plates containing 1.5% agar or in flasks with constant shaking at 150 rpm at 25 °C under 16:8 h of light/dark photoperiod with 54 μmol photons m^{-2} s^{-1} light intensity.

Scenedesmus sp. ME02 was mixotrophically cultivated by inoculating 2-3 x 10^5 cells mL^{-1} in 1 L of TAP:BG-11 (1:1 v/v) medium in 2-L Erlenmeyer flasks and the microalga was grown with constant shaking at 150 rpm at 25 °C under 16:8 h of light/dark photoperiod until cells reached late logarithmic growth phase and harvested at seven days after cultivation.

Hindakia tetrachotoma was mixotrophically cultivated by inoculating 2-3 x 10^5 cells mL^{-1} in 1 L of TAP medium in 2-L Erlenmeyer flasks and the microalga was grown with constant shaking at 150 rpm at 25 °C under 16:8 h of light/dark photoperiod until cells reached late logarithmic growth phase and harvested at six days after cultivation.

Micractinium sp. ME05 was cultivated under both mixotrophic and heterotrophic growth conditions. For mixotrophic cultivation, 2-3 x 10^5 cells mL^{-1} of *Micractinium* sp. ME05 were inoculated in 1 L of TAP medium in 2-L Erlenmeyer flasks and grown with constant shaking at 150 rpm at 25 °C under 16:8 h of light/dark photoperiod until cells reached late logarithmic growth phase and harvested at three days after cultivation. Heterotrophic cultivation was performed by inoculating 2-3 x 10^5 cells mL^{-1} *Micractinium* sp. ME05 into 1 L bold's basal medium (BBM) supplemented with 19 g of molasses hydrolysate in 2-L Erlenmeyer flasks. Then,

Micractinium sp. ME05 cells were grown under complete darkness at 30 °C by supplying air at a flow rate of 0.5 L min^{-1} through an aquarium pump and harvested after 5 days of incubation. The composition of BBM and molasses, preparation of molasses hydrolysate and the optimum molasses hydrolysate amount for the highest biomass concentration were previously described by Engin et al. (2018a).

Time points for harvesting of all three microalgal species marked the highest biomass concentration obtained. The growth characteristics of *Scenedesmus* sp. ME02, *Micractinium* sp. ME05 and *Hindakia tetrachotoma* under the indicated conditions were previously reported in Onay et al. (2014), Sonmez et al. (2016) and Engin et al. (2018a). The compositions of growth media, TAP, BG-11 and BBM, are given in Appendix A.

3.4   Preparation of microalgal extracts

The microalgal cells were grown as described above and harvested by centrifugation at 3600x g for 20 min at 4 °C (Sorvall RC 5C Plus, GMI Inc, USA). The collected pellet was frozen at -80 °C overnight and freeze-dried using a lyophilizer (BW-10B, Shanghai Bluewave Industry, China). Four different solvents; ethanol:water mixture (3:1 v/v), ethyl acetate, hexane and water were used for the extraction of freeze-dried *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* cells. For the extraction of *Micractinium* sp. ME05 cells cultivated either mixotrophically or heterotrophically, six different solvents; methanol, ethanol, acetone, hexane, ethyl acetate and water were used.

For preparation of the microalgal extracts, 0.2 g of freeze-dried microalgal biomass was extracted with 5 mL of solvent at room temperature by sonication in an ultrasonic water bath (Elmasonic S 120H, Elma Schmidbauer GmbH, Germany) for 20 min followed by stirring on an orbital shaker (KS 4000 i control, IKA-Werke GmbH, Germany) for 1 h. Then, the extract was centrifuged at 3800x g for 10 min (Rotina 420, Hettich, Germany), and the aqueous phase was collected in separate bottles. The residual pellets were re-suspended in the same solvents and re-extracted

following the same procedure as mentioned above. The obtained extracts were combined and filtered through a 0.45 µm pore-sized polytetrafluoroethylene (PTFE) syringe filter. Finally, the extracts were dried using a rotary evaporator (Hei-VAP Precision, Heidolph Instruments, Germany) until the solvents were completely removed. The dried residues were weighed for calculation of extraction yields, then re-suspended in methanol to a concentration of 10 mg mL^{-1} and stored at -20 °C for further analysis.

3.5   Determination of total phenolic content

Total phenolic content of the microalgal biomass extracted in the above-mentioned solvents was determined by the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). Each sample at a volume of 100 µL was mixed with 400 µL of Folin-Ciocalteu reagent (1:10 diluted in ultrapure water) in a 2-mL microtube. The mixture was vortexed thoroughly and allowed to stand at room temperature for 5 min. Then, 500 µL of 7.5% $NaNO_2$ (w/v) solution was added to the microtube. After incubating for 1.5 h in the dark at room temperature, 200 µL of sample was transferred to a clear 96-well microplate and the absorbance of each well was measured at 760 nm using a UV–vis microplate reader (Synergy H1, Biotek, USA). A standard curve prepared with serially diluted gallic acid solutions ranging from 10 to 400 mg L^{-1} was used for calibration. Amount of total phenolics in the extracts was calculated as gallic acid equivalents using the regression equation of the standard curve. Total phenolic content was expressed as mg gallic acid equivalents per gram dry weight of sample (mg GAE g^{-1} DW).

3.6   Determination of total flavonoid content

Total flavonoid content of the microalgal extracts were determined using the aluminum chloride colorimetric method (Zhishen et al. 1999). One milliliter of extract was mixed with 4 mL of ultrapure water in a 20-mL glass tube and 0.3 mL

of 5% (w/v) $NaNO_2$ was added the sample. After 5 min of incubation at ambient temperature, the sample was mixed with 0.3 mL of 10% (w/v) $AlCl_3$ (prepared in ethanol). The test tube was vortexed thoroughly and allowed to stand at room temperature for 6 min. Then, 2 mL of 1 M NaOH solution was added and the total volume was adjusted to 10 mL by adding 2.4 mL of ultrapure water. After vortexing briefly, 200 µL of sample was transferred to a clear 96-well microplate and the absorbance of each well was measured at 510 nm using a UV–vis microplate reader (Synergy H1, Biotek, USA). A standard curve was also prepared using serial quercetin solutions ranging from 5 to 100 mg L^{-1}. Total flavonoid content of the microalgal extracts was calculated using the regression equation of the standard curve prepared with quercetin and results were expressed as mg quercetin equivalents per gram dry weight of sample (mg QE g^{-1} DW).

3.7   Determination of total carotenoid content

Amount of total carotenoids in the microalgal extracts was calculated by the spectrophotometric method described by Lichtenthaler and Buschmann (2001). Absorbance of the microalgal extracts in pure methanol was recorded at 470, 652 and 665 nm using a UV–vis spectrophotometer (Synergy H1, Biotek, USA) and total carotenoid content was calculated according to the Lichtenthaler equations as following:

c_a (µg mL^{-1}) = 16.72 A_{665} – 9.16 A_{652}
c_b (µg mL^{-1}) = 34.09 A_{652} – 15.28 A_{665}
$c_{(x+c)}$ (µg mL^{-1}) = (1000 A_{470} – 1.63 c_a – 104.96 c_b)/221

Where c_a and c_b are concentrations of chlorophyll a and b, respectively, and $c_{(x+c)}$ is the concentration of total carotenoids. The results were expressed as mg carotenoid per gram dry weight of sample (mg carotenoid g^{-1} DW).

3.8 Evaluation of total antioxidant capacity

The antioxidant capacity of microalgal extracts was measured using two different colorimetric antioxidant assays, namely; 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. The synthetic antioxidant compound 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid also named as Trolox was also used as a standard for preparation of the calibration curve in both assays.

The microalgal extracts at various concentrations ranging from 50 to 2000 µg mL^{-1} were used for the measurement of total antioxidant capacity. For this, main stocks of the microalgal extracts at concentration of 10 mg mL^{-1} was initially diluted to 2 mg mL^{-1} concentration in methanol. Then, the microalgal extracts were serially diluted as described in Table 3.1 in order to obtain the working concentration range.

Table 3.1 Serial dilution of the microalgal extracts in methanol.

Tube	Volume of methanol (µL)	Volume and source of the extract	Final concentration (µg mL^{-1})
A	0	600 µL of stock	2000
B	250	750 µL of stock	1500
C	600	600 µL of stock	1000
D	300	300 µL of Tube B	750
E	300	300 µL of Tube C	500
F	300	300 µL of Tube E	250
G	300	300 µL of Tube F	125
H	300	200 µL of Tube G	50

3.8.1 DPPH radical scavenging assay

DPPH$^{\bullet}$ radical scavenging activity was performed according to the microplate assay described by Cheng et al. (2006). The stock solution of DPPH$^{\bullet}$ radical was prepared at 5 mM concentration by dissolving 5.12 mg of DPPH in 2.6 mL of methanol and the working solution was freshly prepared at 0.2 mM concentration by dilution of the stock in methanol. Then, 100 µL of microalgal extracts at concentrations ranging from 50 to 2000 µg mL^{-1} in methanol was mixed with 100 µL of 0.2 mM DPPH$^{\bullet}$ solution in a clear 96-well microplate. After incubating for 30 min at room temperature in the dark, absorbance of each well was recorded at 515 nm using a microplate reader (Synergy H1, Biotek, USA). The percentage of scavenged DPPH$^{\bullet}$ radical was calculated according to the following equation:

DPPH$^{\bullet}$ scavenging activity (%) = [1-(*As-Asc*)/*Ac*]*100

Where As is absorbance of the sample (100 µL of sample with 100 µL of DPPH$^{\bullet}$ radical solution), Asc is absorbance of the sample control (100 µL of sample with 100 µL of methanol) and Ac is absorbance of the control (100 µL of methanol with 100 µL of DPPH$^{\bullet}$ radical solution). A standard curve was also prepared with serially diluted Trolox solutions in the range of 2.5 to 80 µmol L^{-1} concentrations. Total antioxidant capacity of the microalgal extracts was calculated as Trolox equivalents using the regression equation of the standard curve. Results were as µmol Trolox equivalents per gram dry weight of sample (µmol TE g^{-1} DW) and (%) DPPH$^{\bullet}$ radical scavenging effect of the extracts at 1 mg mL^{-1} concentration.

3.8.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant capacity of the extracts was also evaluated by FRAP assay through monitoring the reduction of Fe^{3+}-TPTZ to blue-colored Fe^{2+}-TPTZ (Firuzi et al. 2005). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (prepared in 40 mM HCl) and 20 mM FeCl$_3$·6H$_2$O. The working FRAP solution was freshly prepared by mixing ten volumes of acetate buffer, one volume of TPTZ

solution and one volume of $FeCl_3 \cdot 6H_2O$ solution, and warmed at 37 °C in a water bath prior to use. Then, 25 µL of the microalgal extracts at various concentrations in the range of 50 to 2000 µg mL^{-1} was mixed with 175 µL of pre-warmed FRAP solution in a clear 96-well microplate. The microplate was allowed to stand at room temperature for 30 min in the dark. Absorbance of each well was measured at 593 nm using a microplate reader (Synergy H1, Biotek, USA). Trolox solutions ranging from 2.5 to 400 µmol L^{-1} were also used for preparation of a standard curve. Total antioxidant capacity of the microalgal extracts was calculated as Trolox equivalents using the regression equation of the standard graph. FRAP values were expressed as µmol Trolox equivalents per gram dry weight of sample (µmol TE g^{-1} DW).

3.9 Reversed Phase High Performance Liquid Chromatography Analysis (RP-HPLC)

Twelve different phenolic compounds, namely; gallic acid, benzoic acid, 4-hydroxy benzoic acid, vanillic acid, syringic acid, cinnamic acid, coumaric acid, caffeic acid, chlorogenic acid, rosmarinic acid, quercetin and rutin were identified in the microalgal extracts by reversed phase HPLC as described in Bulut et al. (2019). The microalgal extracts at a concentration of 1000 ppm was passed through a 0.45 µm PTFE syringe filter prior to injection. Reversed phase HPLC analysis was performed with a Waters Alliance 2695 series HPLC system equipped with Waters 2489 UV/Vis detector (Waters Corporation, Milford, MA, USA). Chromatographic separation was conducted on an ACE 5 C$_{18}$ analytical column (250 x 4.6 mm) with 5 µm packing material (Advanced Chromatography Technologies Ltd, Scotland). Elution was carried out with a gradient pump mode involving three mobile phases; mobile phase A: 2% (v/v) acetic acid, mobile phase B: acetonitrile and 0.5% (v/v) acetic acid (1:1 v/v) and mobile phase C: acetonitrile. The gradient was set as following: 0-8 min: 95% A and 5% B; 8-10 min: 80% A and 20% B; 10-17 min: 78% A and 22% B; 17-19 min: 75% A and 25% B; 19-30 min: 73% A and 27% B; 30-35 min: 60% A and 40% B; 35-40 min: 55% A and 45% B; 40-45 min: 35% A

and 65% B; 56-50 min: 10% B and 90% C; 50-52 min: 100% C; and 52-60 min: 95% A and 5% B. The flow rate was 1.2 mL min^{-1}, the injection volume was 20 µL and the column temperature was maintained at ambient temperature. Simultaneous monitoring was done using a UV/Vis detector with reference wavelength of 280 nm. Retention times and peak areas of both authentic standards and microalgal extracts were monitored automatically by Empower 3 Chromatography Data Software (Waters Corporation, USA). Amount of individual phenolic compounds was quantified by comparison of the chromatographic peaks in the microalgal extracts with those of authentic standards using the same reversed phase HPLC operating conditions.

3.10   Mammalian cell lines and cell culture

Human breast adenocarcinoma cell line (MCF-7) and human cervical cancer cell line (HeLa) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU mL^{-1}) and streptomycin (100 µg mL^{-1}) in a CO_2 incubator with 5% CO_2 at 37 °C.

3.10.1   Sub-culturing

Cells maintained in T75 cell culture flasks were routinely sub-cultured in order to obtain a proper cell stage and supply the sufficient nutrients required for growth. When the cells reached 80% confluence, growth medium was removed from the culture flask and cells were washed with pre-warmed 10 mL of PBS. Then, the cells were incubated in 4 mL of trypsin-EDTA solution at 37 °C for 5 min in order to dissociate the cell-to-cell and cell-to-flask surface interactions. Six milliliters of fresh complete medium was added to the culture flask and detached cells harvested by centrifugation at 200x g for 5 min at ambient temperature (Rotina 420, Hettich,

Germany). The cell pellet was re-suspended in fresh complete medium, an aliquot of the cell suspension was passed into a new culture flask and total volume of the flask was adjusted to 10 mL with addition of fresh medium.

3.10.2   Cell freezing and thawing

Cells were frozen for long-term storage using the cryopreservation method described by Freshney (2015). For this, cells were harvested by trypsinization as described in the sub-culturing section and the cell suspension was centrifuged at 200x g for 5 min at ambient temperature. Supernatant was discarded and cell pellet was re-suspended in the complete medium containing 10% of DMSO to a concentration of 5 x 10^6 to 1 x 10^7 cells mL^{-1}. Then, cells were aliquoted into cryogenic storage vials and immediately frozen at -20 °C for 1 h and -80 °C for 24 h. For long-term storage, cryogenic vials were transferred to liquid nitrogen storage.

In order to thaw the cryopreserved cells, cryogenic vials containing the frozen cells were removed from the liquid nitrogen storage and immediately placed into a 37 °C water bath. Cells were completely thawed by gently swirling the vial in the water bath and then transferred into 4 mL of pre-warmed complete medium. The cell suspension was centrifuged at 200x g for 5 min at room temperature. After removal of supernatant, the cell pellet was re-suspended in 4 mL of complete medium by gently pipetting and seeded into a T25 cell culture flask. Finally, cells were transferred into T75 cell culture flasks after reaching 80% confluence.

3.10.3   Cell counting

Cells were routinely counted for properly sub-culturing and seeding for further experiments. To this end, trypan blue staining was performed using a hemocytometer. Cells at 80% confluence were harvested and re-suspended in complete medium as described in sub-culturing section. Then, 90 μL of the cell suspension was mixed with 10 μL of 0.4% (w/v) trypan blue solution and allowed to

stand at room temperature for 2 min. The stained cell suspension was loaded onto a Neubauer hemocytometer (Hausser Scientific, USA). The number of viable (brownish) and non-viable (blue) was counted using a phase-contrast microscope (Olympus, USA) and cell concentrations were calculated according to the following formula:

Concentration (cells mL^{-1}) = Number of cells counted per square millimeter x dilution factor x 10^6

3.11   Cell viability assay

Effect of the microalgal extracts on viability of MCF-7 and HeLa cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann (1983). MCF-7 and HeLa cells were seeded in a 96-well cell culture plate at a density of 1.5 x 10^4 cells per well at 100 μL volume. After incubation in an incubator with 5% CO_2 at 37 °C for 24 h, the cell culture medium was removed and cells were washed with pre-warmed PBS. Fresh medium containing various concentrations of the microalgal extracts (ranging from 12.5 to 400 μg mL^{-1}) was added and cells were incubated for 48 h under the same conditions. Then, the cells were treated with 10 μL of MTT solution (prepared in PBS at 5 mg mL^{-1} concentration) for 4 h. Subsequently, 100 μL of 10% (w/v) SDS solution acidified with 0.01 M of HCl was added into each well and incubated overnight at 37 °C in order to solubilize the formazan. Absorbance of each well was measured at 570 nm using a microplate reader (Synergy H1, Biotek, USA). The viability of negative control cells was accepted as 100% and the relative viability of cell treated with various concentrations of the microalgal extracts was calculated accordingly.

3.12   Measurement of intracellular reactive oxygen species (ROS) generation

Inhibitory effects of the microalgal extracts on H_2O_2-induced reactive oxygen species (ROS) generation was evaluated using the cell-permeant probe 2',7'-

dichlorodihydrofluorescein diacetate (H$_2$DCFDA) according to the method described by Zhuang et al. (2017) with slight modifications. MCF-7 cells at a density of 1 x 10^5 cell mL^{-1} were seeded in a 96-well black cell culture plate with 200 μL of culture medium and incubated for 24 h at 37 °C with 5% CO$_2$. Then, cells were treated with the microalgal extracts in a concentration range of 50-400 μg mL^{-1} for 48 h. Control groups included the cells treated with the culture medium containing vehicle solution and 8 μg mL^{-1} of ascorbic acid as negative and positive controls, respectively. After incubation, growth medium was discarded and cells were exposed to 0.5 mM of H$_2$O$_2$ for 6 h in order to induce oxidative stress. After the treatment, cells were washed twice with pre-warmed PBS and incubated with 20 μM of H$_2$DCFDA for 30 min in the dark at 37 °C. Subsequently, cells were immediately washed twice and re-suspended in PBS. Formation of the fluorescent 2',7'-dichlorofluorescein (DCF) due to oxidation of the non-fluorescent H$_2$DCFDA was monitored with an excitation wavelength of 495 nm and an emission wavelength of 525 nm using a fluorescence microplate reader (Synergy H1, Biotek, USA).

3.13   Apoptosis assay

Cytoprotective activity of the microalgal extracts against H$_2$O$_2$-induced apoptosis of MCF-7 cells was measured by an Annexin V-FITC/PI apoptosis assay kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. MCF-7 cells at a density of 0.3 x 10^6 cell mL^{-1} were seeded in a 6-well cell culture plate with 1 mL of culture medium and incubated for 24 h at 37 °C with 5% CO$_2$. After adherence of the cells, culture medium was replaced with fresh medium containing the microalgal extracts at 400 μg mL^{-1} concentration and further incubated for 48 h under the same conditions. Control cells were added with culture medium containing the corresponding vehicle solution. After incubation, oxidative stress was induced by exposing the cells to 1 mM of H$_2$O$_2$ for 4 h. Cells were harvested by trypsinization and washed with PBS twice. Then, the cell pellet was re-suspended in 200 μL of 1X binding buffer, which is followed by staining with 5 μL of Annexin V-FITC (20 μg

mL^{-1} in Tris-NaCl) for 15 min at room temperature and 10 µL of propidium iodide (PI) (50 µg mL^{-1} in 1X binding buffer) for 10 min in an ice bath in the dark. After incubation, cells were immediately analyzed using a flow cytometer (BD Accuri C6, BD Biosciences, USA).

3.14   Statistical analysis

All analyses were performed in triplicate and results were expressed as mean ± standard error (SE). Mean values of the data were analyzed by analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test in order to determine the differences between mean values. Statistical significance was determined at 5% level ($p < 0.05$). Correlations among different assays were calculated using Pearson's correlation coefficient (r). All statistical analyses were carried out using R software version 3.4.2 (R Core Team 2013).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction yields

The solubility of antioxidants, which depends mainly on the polarity of the solvent used is a major determinant in accurate assessment of antioxidant capacity. In addition to their diverse chemical structures, antioxidants such as phenolic compounds are also found attached to sugars or proteins *in vivo*, which further affects their solubility. Therefore, finding a single solvent that solubilizes all the compounds of interest simultaneously is a challenge (Farvin and Jacobsen, 2013).

4.1.1 Extraction yields of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03

In this study, different solvents with varying polarity were used in order to determine the maximum yield. Four different solvents; ethanol/water mixture (3:1 *v/v*), ethyl acetate, hexane and water were used for extraction of antioxidants from *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03. The extraction yields are given in Table 4.1. In *Scenedesmus* sp. ME02, the highest extraction yield of 23.98 ± 2.47% was obtained with ethanol/water which is used to extract both polar and nonpolar compounds followed by 15.32 ± 0.18% and 8.47 ± 0.55% extraction yields for water and hexane, respectively. The lowest extraction yield of 5.42 ± 1.06% was obtained for ethyl acetate, which has medium polarity. In *Hindakia tetrachotoma* ME03, the highest extraction yield of 54.11 ± 2.45% was observed with water which was followed by 36.32 ± 2.69% and 10.21 ± 0.80 extraction yields for ethanol/water and

ethyl acetate, respectively. Surprisingly, the lowest extraction yield of $5.56 \pm 1.70\%$ was obtained for hexane, which has the lowest polarity.

Table 4.1 Extraction yields of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 extracts prepared using different solvents.

	Extraction yield (%)	
Solvent	***Scenedesmus* sp. ME02**	***H. tetrachotoma* ME03**
Ethanol/water	23.98 ± 2.47	36.32 ± 2.69
Ethyl acetate	5.42 ± 1.06	10.21 ± 0.80
Hexane	8.74 ± 0.55	5.56 ± 1.70
Water	15.32 ± 0.18	54.11 ± 2.45

Results are expressed as mean $\pm$ standard error of three measurements (n=3).

4.1.2   Extraction yield of *Micractinium* sp. ME05

In order to extract antioxidants from either mixotrophically or heterotrophically grown *Micractinium* sp. ME05, six different solvents, namely; methanol, ethanol, acetone, hexane, ethyl acetate and water were used. The extraction yields are given in Table 4.2. The highest extraction yield of $30.40 \pm 0.94\%$ was obtained in methanol followed by $26.16 \pm 1.08\%$ in water for mixotrophic growth. For heterotrophic growth, the highest yield was equal in methanol ($38.23 \pm 3.90\%$) and water ($38.33 \pm 0.34\%$). The lowest extraction yield was in ethyl acetate ($11.54 \pm 1.47\%$) and acetone ($8.29 \pm 1.41\%$) for mixotrophic and heterotrophic cultivation, respectively. The differences in extraction yields of methanol and water with respect to acetone, ethyl acetate, ethanol and hexane were highly significant for both mixotrophic and heterotrophic growth conditions ($p < 0.001$).

These results are consistent with previous studies, which suggest that the choice of solvent significantly effects the yield of extractable substances and higher extraction yields were obtained with polar solvents (Goiris et al. 2012; Machu et al. 2015; Jerez-Martel et al. 2017).

Table 4.2 Extraction yields of *Micractinium* sp. ME05 extracts prepared using different solvents and growth conditions.

	Extraction yield (%)	
Solvent	**Mixotrophic growth**	**Heterotrophic growth**
Methanol	30.40 ± 0.94	38.23 ± 3.90
Ethanol	14.85 ± 1.88	17.78 ± 4.78
Acetone	16.46 ± 0.95	8.29 ± 1.41
Hexane	12.70 ± 2.62	15.45 ± 2.00
Ethyl acetate	11.54 ± 1.47	13.92 ± 1.31
Water	26.16 ± 1.08	38.33 ± 0.34

Results are expressed as mean $\pm$ standard error of three measurements (n=3).

4.2   Total phenolic content of microalgal extracts

Phenolic compounds are considered to be the major contributors of antioxidant activity in plants (Dai and Mumper, 2010), although there are conflicting results on the correlation of antioxidant activity and total phenolic content of microalgal samples (Li et al. 2007; Goiris et al. 2012; Shetty and Sibi, 2015). Phenolic content of the extracts depends highly on the type of solvent used. Therefore, total phenolic contents of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 extracted separately in different solvents with different polarity were compared. The amount of total phenolic compounds in extracts were calculated using a standard curve prepared with serially diluted gallic acid solutions ranging from 10 to 400 mg L^{-1}, and the results were expressed as mg gallic acid equivalents per gram dry weight of sample (mg GAE g^{-1} DW). The standard curve of gallic acid is given in Appendix B.

4.2.1 Total phenolic contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03

Total phenolic contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 extracts prepared in four different solvents are represented in Table 4.3. In *Scenedesmus* sp. ME02, the highest total phenolic content of 5.40 ± 0.28 mg GAE g^{-1} DW was recorded in ethanol/water, followed by 3.73 ± 0.65 mg GAE g^{-1} DW in ethyl acetate, 1.97 ± 0.03 mg GAE g^{-1} DW in water and 1.13 ± 0.11 mg GAE g^{-1} DW in hexane. Similarly, total phenolic content of samples was measured in a decreasing order as ethanol/water>ethyl acetate>water>hexane in *Hindakia tetrachotoma* ME03, as well. Despite, the extracts of *Hindakia tetrachotoma* ME03 showed the similar order according to the nature of the solvent, total phenolic contents were significantly higher than those of *Scenedesmus* sp. ME02. The highest total phenolic content of 18.30 ± 0.72 mg GAE g^{-1} DW was measured in ethanol/water, followed by 7.75 ± 0.56 mg GAE g^{-1} DW in ethyl acetate, 4.91 ± 0.70 mg GAE g^{-1} DW in water and 3.21 ± 0.17 mg GAE g^{-1} DW in hexane. The presence of phenolics in higher amounts as extracted by the polar solvents compared to the non-polar hexane is consistent with previous data and can be explained by the largely polar nature of the phenolic compounds in microalgae (Hajimahmoodi et al. 2010; Goiris et al. 2012).

Scenedesmus sp. ME02 and *Hindakia tetrachotoma* ME03 exhibit a relatively high total phenolic content among previously studied microalgae. Goiris et al. (2012) screened different microalgal biomass samples for their total phenolic content using the same solvents as the present study. Similarly, the highest total phenolic content was found to be in ethanol/water with a range of 0.5 to 4.6 mg GAE g^{-1} DW. Three out of thirty-two microalgae with total phenolics of 3 mg GAE g^{-1} DW or higher in ethanol/water were considered to have a relatively high phenolic content. A *Scenedesmus obliquus* strain that was among the microalgae studied contained 1.94 mg GAE g^{-1} DW. Yet in another study by Custódio et al. (2014), total phenolic content of a different *Scenedesmus* sp. was determined to be 0.05 mg GAE g^{-1} DW

in hexane compared to 1.13 mg GAE g^{-1} DW in *Scenedesmus* sp. ME02 and 3.21 mg GAE g^{-1} DW in *Hindakia tetrachotoma* ME03. It is important to note that many factors including growth medium, pH, temperature, light intensity, harvest time in addition to the natural variability and the extraction procedure effect the phenolic content and antioxidant capacities of microalgae (Guedes et al. 2011; Shetty and Sibi 2015; Aremu et al. 2016). For instance, Guedes et al. (2011) reported that the antioxidant compounds studied in *Scenedesmus obliquus* strain M2-1 highly varied under different temperature and pH. The lowest total phenolic contents recorded in *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 was in hexane extracts; 1.13 mg GAE g^{-1} DW and 3.21 mg GAE g^{-1} DW, respectively. Aremu et al. (2016) reported that the harvest time, nitrogen levels and choice of microalgae had a significant impact on the phenolic content. A single time point was selected for harvesting in the present study, to maximize the microalgal biomass for subsequent analysis; however, different harvest times may as well affect the antioxidant capacities and total phenolic contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03, and can be assessed in future studies.

Table 4.3 Total phenolic contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 extracts.

	Total phenolic content (mg GAE g^{-1} DW)	
Solvent	*Scenedesmus* **sp. ME02**	*H. tetrachotoma* **ME03**
Ethanol/water	5.40 ± 0.28	18.30 ± 0.72
Ethyl acetate	3.73 ± 0.65	7.75 ± 0.56
Hexane	1.13 ± 0.11	3.21 ± 0.17
Water	1.97 ± 0.03	4.91 ± 0.70

Results are mean ± standard error of three measurements (n=3).

4.2.2   Total phenolic content of *Micractinium* sp. ME05

Total phenolic contents of *Micractinium* sp. ME05 grown under two different growth conditions and extracted in six different solvents are given in Table 4.4. The highest phenolic content was recorded in methanol extracts as 18.11 ± 2.17 mg GAE g^{-1} DW and 11.47 ± 1.41 mg GAE g^{-1} DW for mixotrophic and heterotrophic growth, respectively. The difference between total phenolic contents of two growth conditions in methanol extracts is very significant. These results are consistent with a previous report in which, both *Chlorella vulgaris* and *Scenedesmus obliquus* had higher phenolic content in mixotrophic than heterotrophic cultures (Shetty and Sibi, 2015). The lowest total phenolic contents of both mixotrophic and heterotrophic cultivation were measured in hexane extracts as 3.87 ± 0.83 mg GAE g^{-1} DW and 2.40 ± 0.19 mg GAE g^{-1} DW, respectively. The difference in results was not statistically significant. Similarly, *Micractinium* sp. ME05 extracted by polar solvents showed significantly higher concentrations of phenolic compounds compared to nonpolar hexane extracts irrespective of the cultivation method (Goiris et al. 2012; Bulut et al. 2019). Safafar et al. (2015) also noted that methanol is the most efficient solvent for extraction of phenolic compounds due to higher solubility as well as higher capability to dissolve cell membranes. Ethyl acetate, water and hexane extracts of mixotrophically cultivated *Micractinium* sp. ME05 contained higher amounts of phenolics compared to the same solvent extracts of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 revealing the differences in composition of these three thermo-tolerant freshwater strains (Bulut et al. 2019).

Table 4.4 Total phenolic contents of *Micractinium* sp. ME05 extracts prepared using different solvents and cultivation modes.

	Total phenolic content (mg GAE g^{-1} DW)	
Solvent	**Mixotrophic growth**	**Heterotrophic growth**
Methanol	18.11 ± 2.17	11.47 ± 1.41
Ethanol	7.40 ± 1.46	6.67 ± 1.07
Acetone	7.37 ± 0.83	4.77 ± 0.35
Hexane	3.87 ± 0.83	2.40 ± 0.19
Ethyl acetate	8.02 ± 0.52	4.76 ± 0.09
Water	6.65 ± 1.32	7.07 ± 0.66

Results are mean ± standard error of three measurements (n=3).

4.3   Total flavonoid content of microalgal extracts

Flavonoids are another important class of natural bioactive compounds; generally considered as the main group of polyphenols and widely found in fruits, vegetables and certain beverages. They are broadly associated with beneficial health effects, particularly antioxidant activity, due to their capacity to absorb UV light, to inhibit the generation of free radicals and to quench formed free radicals (Brunetti et al. 2013). Similar to phenolic compounds, the extraction of flavonoids mainly depends on the type and polarity of solvent system used. Thus, total flavonoid contents of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 extracted separately in different solvents with different polarity were compared. Total flavonoid content of the extracts were calculated using a standard curve prepared with serially diluted quercetin solutions ranging from 5 to 100 mg L^{-1}, and the results were expressed as mg quercetin equivalents per gram dry weight of sample (mg QE g^{-1} DW). The standard curve of quercetin is given in Appendix C.

4.3.1 Total flavonoid contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03

Total flavonoid contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 extracts prepared in four different solvents are represented in Table 4.5. The total flavonoid content of *Scenedesmus* sp. ME02 was found to be 1.61 ± 0.76 mg QE g^{-1} DW and 0.93 ± 0.30 mg QE g^{-1} DW in ethanol/water and ethyl acetate, respectively. Hexane and water extracts did not contain any detectable levels of flavonoids. In *Hindakia tetrachotoma* ME03, the highest total flavonoid content of 2.93 ± 0.54 mg QE g^{-1} DW was surprisingly obtained in water extracts, followed by 1.68 ± 0.17 mg QE g^{-1} DW in ethyl acetate, 1.62 ± 0.19 mg QE g^{-1} DW in ethanol/water and 0.08 ± 0.02 mg QE g^{-1} DW in hexane extracts.

Table 4.5 Total flavonoid contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 extracts

	Total flavonoid content (mg QE g^{-1} DW)	
Solvent	*Scenedesmus* sp. ME02	*H. tetrachotoma* ME03
Ethanol/water	1.61 ± 0.76	1.62 ± 0.19
Ethyl acetate	0.93 ± 0.30	1.68 ± 0.17
Hexane	ND[a]	0.08 ± 0.02
Water	ND	2.93 ± 0.54

Results are mean $\pm$ standard error of three measurements (n=3). [a] Not detected.

4.3.2 Total flavonoid content of *Micractinium* sp. ME05

Total flavonoid contents of *Micractinium* sp. ME05 grown under two different growth modes and extracted in six different solvents are represented in Table 4.6. For mixotrophic growth conditions, the highest total flavonoid content of *Micractinium* sp. ME05 was found in methanol extracts (5.72 ± 0.26 mg QE g^{-1} DW) followed by ethanol extracts (5.21 ± 1.70 mg QE g^{-1} DW). Similarly, flavonoid content of heterotrophically grown cell extracts was highest in methanol with a

concentration of 3.22 ± 0.27 mg QE g^{-1} DW. The difference in total flavonoid amounts of methanol extracts between mixotrophic and heterotrophic cultivation was not statistically significant. However, there was a significant reduction in flavonoid content of ethanol extracts of heterotrophic samples (1.89 ± 0.35 mg QE g^{-1} DW) compared to ethanol extracts of mixotrophic microalgae (5.21 ± 1.70 mg QE g^{-1} DW).

Table 4.6 Total flavonoid contents of *Micractinium* sp. ME05 extracts prepared using different solvents and cultivation modes.

	Total flavonoid content (mg QE g^{-1} DW)	
Solvent	**Mixotrophic growth**	**Heterotrophic growth**
Methanol	5.72 ± 0.26	3.22 ± 0.27
Ethanol	5.21 ± 1.70	1.89 ± 0.35
Acetone	4.21 ± 0.68	1.40 ± 0.16
Hexane	1.07 ± 0.17	0.86 ± 0.07
Ethyl acetate	2.39 ± 0.08	2.09 ± 0.45
Water	1.15 ± 0.07	1.47 ± 0.02

Results are mean $\pm$ standard error of three measurements (n=3).

Despite the health benefits of flavonoids in terms of their antioxidant potential is well recognized in plants, relatively few studies to date investigated the total flavonoid content of microalgae (Panche et al. 2016). Recently, Goiris et al. (2014) determined the presence of distinct classes of flavonoids in different evolutionary lineages of microalgae including *Chlorophyta* using ultra-high performance liquid chromatography-two-dimensional mass spectrometry (UHPLC-MS/MS) analysis and demonstrated that different classes of microalgae contained a diverse range of flavonoids although in smaller quantities compared to plants. Also, Safafar et al. (2015) reported the total flavonoid content of methanolic extracts of seven different microalgae strains to be in the range of 0.84 ± 0.12 to 4.03 ± 1.10 mg QE g^{-1} DW. Total flavonoid content of these three thermo-tolerant freshwater strains, particularly *Micractinium* sp. ME05, is higher than most of other microalgae reported previously.

Therefore, results in this study contribute to the limited number of studies in literature on the presence and quantification of flavonoids in microalgae.

4.4   Total carotenoid content of microalgal extracts

One of the main constituents of microalgal cells is colored pigments, chlorophylls and carotenoids, which participate in photosynthetic pathways of microalgae by absorbing and reflecting the visible light at certain wavelengths. In particular, carotenoids are involved in vital processes such as light harvesting, photo-protection, structure stabilization and single oxygen scavenging. Due to their light harvesting properties, chlorophylls (Chl a and Chl b) and carotenoids can be quantitatively determined in crude extracts by UV-Vis spectroscopy. Although photosynthetic pigments absorb light in overlapping spectral regions, certain methods enable the accurate quantitative determination of Chl a, Chl b and total carotenoids by applying equations (Dere et al. 1998; Lichtenthaler and Buschmann, 2001).

4.4.1   Total carotenoid contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03

The spectroscopic analysis showed that the total carotenoid content of *Scenedesmus* sp. ME02 extracts was 0.61 ± 0.05 mg g^{-1} DW, 0.80 ± 0.32 mg g^{-1} DW and 0.15 ± 0.02 mg g^{-1} DW for ethanol/water, ethyl acetate and hexane extracts, respectively. No carotenoids were detected in the water extract (Table 4.7). Although the highest carotenoid content was obtained in ethyl acetate, the difference was not statistically significant among any of the three solvents. The total carotenoid contents of *Hindakia tetrachotoma* ME03 were measured as 1.18 ± 0.06 mg g^{-1} DW, 0.75 ± 0.04 mg g^{-1} DW, 0.11 ± 0.01 mg g^{-1} DW and 0.12 ± 0.04 mg g^{-1} DW for ethanol/water, ethyl acetate, hexane and water extracts, respectively. The difference between ethanol/water and other solvents was statistically significant. Even tough, total carotenoid content of *Scenedesmus* sp. ME02 is similar to that of *Scenedesmus*

obliquus (0.44 mg g^{-1} DW in ethanol/water) studied previously by Goiris et al. (2012), is in the lower range in comparison to other microalgae. However, *Hindakia tetrachotoma* ME03 was found to have slightly higher amounts of total carotenoids as compared to other microalgae.

Table 4.7 Total carotenoid contents of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 extracts

	Total carotenoid content (mg g^{-1} DW)	
Solvent	***Scenedesmus* sp. ME02**	***H. tetrachotoma* ME03**
Ethanol/water	0.61 ± 0.05	1.18 ± 0.06
Ethyl acetate	0.80 ± 0.32	0.75 ± 0.04
Hexane	0.15 ± 0.02	0.11 ± 0.01
Water	ND[a]	0.12 ± 0.04

Results are mean ± standard error of three measurements (n=3). [a] Not detected.

4.4.2   Total carotenoid content of *Micractinium* sp. ME05

Total carotenoid content of *Micractinium* sp. ME05 was measured in methanol, acetone and ethyl acetate extracts as 2.27 ± 0.18 mg g^{-1} DW, 3.02 ± 0.11 mg g^{-1} DW and 3.17 ± 0.21 mg g^{-1} DW, respectively under mixotrophic cultivation (Table 4.8). Total carotenoids of heterotrophically grown microalgae was recorded as 1.65 ± 0.01 mg g^{-1} DW in methanol, 0.32 ± 0.05 mg g^{-1} DW in acetone and 0.32 ± 0.06 mg g^{-1} DW in ethyl acetate extracts. The difference in carotenoid content between mixotrophic and heterotrophic growth was statistically significant for ethyl acetate extracts ($p < 0.05$). To the best of our knowledge, this is the first study that compares the total carotenoid content of microalgae grown under different cultivation modes. Carotenoids in microalgae are involved in protection of the chlorophylls from damaging effects of light exposure through ROS scavenging (Sathasivam and Ki, 2018). These results that show higher carotenoid accumulation in mixotrophic microalgae compared to heterotrophically grown cultures support this role of carotenoids in relation to light exposure.

Table 4.8 Total carotenoid contents of *Microactinium* sp. ME05 extracts prepared using different solvents and cultivation modes.

	Total carotenoid content (mg g^{-1} DW)	
Solvent	Mixotrophic growth	Heterotrophic growth
Methanol	2.27 ± 0.18	1.65 ± 0.01
Ethanol	NT[a]	NT
Acetone	3.02 ± 0.11	0.32 ± 0.05
Hexane	NT	NT
Ethyl acetate	3.17 ± 0.21	0.32 ± 0.06
Water	NT	NT

Results are mean $\pm$ standard error of three measurements (n=3). [a] Not tested.

4.5 Antioxidant capacity of microalgal extracts

The antioxidant capacity of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Microactinium* sp. ME05 was measured by two different assays, namely; DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. Both DPPH and FRAP assays have been successfully employed to measure the antioxidant activities of many fruits as well as macro- and microalgae (Prior et al. 2005). In principle, DPPH assay measures the reducing capacity of tested antioxidants toward the DPPH˙ radical either by direct reduction via electron transfer or by radical quenching via H atom transfer (HAT). FRAP assay, on the other hand, detects the ability of antioxidants to transfer an electron to reduce Fe(III) to Fe(II), which is known as the single electron transfer (SET) mechanism but may be limited by the reactivity of the antioxidants exhibited at different time points (Prior et al. 2005). To assess the reactivity of a broad range of antioxidants including caffeic acid, ascorbic acid and quercetin in the extracts, the measurements of the FRAP assay were followed by a thirty-minute dark incubation in this study.

FRAP assay results are expressed as micromoles Trolox equivalent (TE) per gram dry weight (g^{-1} DW) of microalgae. DPPH assay results, on the other hand, are

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expressed both as % DPPH radical scavenging activity of microalgal extracts at 1 mg mL^{-1} concentration and as micromoles Trolox equivalent (TE) per gram dry weight (g^{-1} DW) of microalgae. The former expression does not factor into account the extraction yield of the samples in each solvent. In order to express the antioxidant capacity of microalgal extracts as Trolox equivalents, a standard curve was prepared with serially diluted Trolox solutions for both DPPH and FRAP assay. The regression equation of the standard curve was used to calculate Trolox equivalents. The standard curves of Trolox for DPPH and FRAP assays are given in Appendix D.

4.5.1 Antioxidant capacities of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03

The results of the antioxidant capacities of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 extracts as % DPPH radical scavenging activity as well as µmol Trolox g^{-1} DW of microalgae are given in Table 4.9 and Table 4.10, respectively.

According to the data obtained by the DPPH and FRAP assays, the highest antioxidant capacity of the cell extracts of *Scenedesmus* sp. ME02 was measured in ethanol/water as 3.71 ± 0.11 µmol TE g^{-1} DW and 47.01 ± 3.14 µmol TE g^{-1} DW, respectively. The extracts in other solvents displayed lower antioxidant capacity. The differences between ethanol/water>ethyl acetate>hexane and water were statistically significant ($p < 0.001$); whereas the difference between hexane and water was not ($p > 0.05$) in both DPPH and FRAP assays. The coefficient of determination (R^2) between the DPPH and FRAP assays was calculated as 0.95 and was highly significant ($p < 0.001$) for all solvents used (Figure 4.1A).

Table 4.9 Antioxidant capacity of *Scenedesmus* sp. ME02 in different solvent extracts determined by DPPH and FRAP assays.

Solvent	(%) DPPH Radical scavenging effect [a]	DPPH (μmol TE g^{-1} DW)	FRAP (μmol TE g^{-1} DW)
Ethanol/water	25.65 $\pm$ 2.58	3.71 $\pm$ 0.11	47.01 $\pm$ 3.14
Ethyl acetate	52.02 $\pm$ 2.61	1.80 $\pm$ 0.25	14.49 $\pm$ 1.14
Hexane	12.99 $\pm$ 1.30	0.60 $\pm$ 0.08	2.85 $\pm$ 0.76
Water	8.40 $\pm$ 0.40	0.61 $\pm$ 0.03	4.30 $\pm$ 0.18

Results are mean $\pm$ standard error of three measurements (n=3). [a] Radical scavenging effects of algal extracts at 1 mg mL^{-1} concentration.

Table 4.10 Antioxidant capacity of *Hindakia tetrachotoma* ME03 in different solvent extracts determined by DPPH and FRAP assays.

Solvent	(%) DPPH Radical scavenging effect [a]	DPPH (μmol TE g^{-1} DW)	FRAP (μmol TE g^{-1} DW)
Ethanol/water	36.67 $\pm$ 1.36	12.42 $\pm$ 1.21	67.98 $\pm$ 3.45
Ethyl acetate	45.89 $\pm$ 2.20	5.51 $\pm$ 0.47	11.96 $\pm$ 2.51
Hexane	50.56 $\pm$ 6.54	2.66 $\pm$ 0.39	7.78 $\pm$ 0.69
Water	12.83 $\pm$ 1.39	5.26 $\pm$ 0.61	8.40 $\pm$ 0.38

Results are mean $\pm$ standard error of three measurements (n=3). [a] Radical scavenging effects of algal extracts at 1 mg mL^{-1} concentration.

Similarly, both DPPH and FRAP assays showed that the highest antioxidant capacity of the extracts of *Hindakia tetrachotoma* ME03 was obtained in ethanol/water mixture as 12.42 $\pm$ 1.21 μmol TE g^{-1} DW and 67.98 $\pm$ 3.45 μmol TE g^{-1} DW, respectively. Consistent with *Scenedesmus* sp. ME02, other solvents displayed relatively lower antioxidant capacity compared to ethanol/water mixture. Moreover, the differences between ethanol/water and other solvents were statistically significant ($p < 0.001$); however the difference between ethyl acetate, hexane and water was not ($p > 0.05$) in both DPPH and FRAP assays. The coefficient of determination (R^2) between the DPPH and FRAP assays was calculated as 0.88 and was highly significant ($p < 0.001$) for all solvents used (Figure 4.1B).
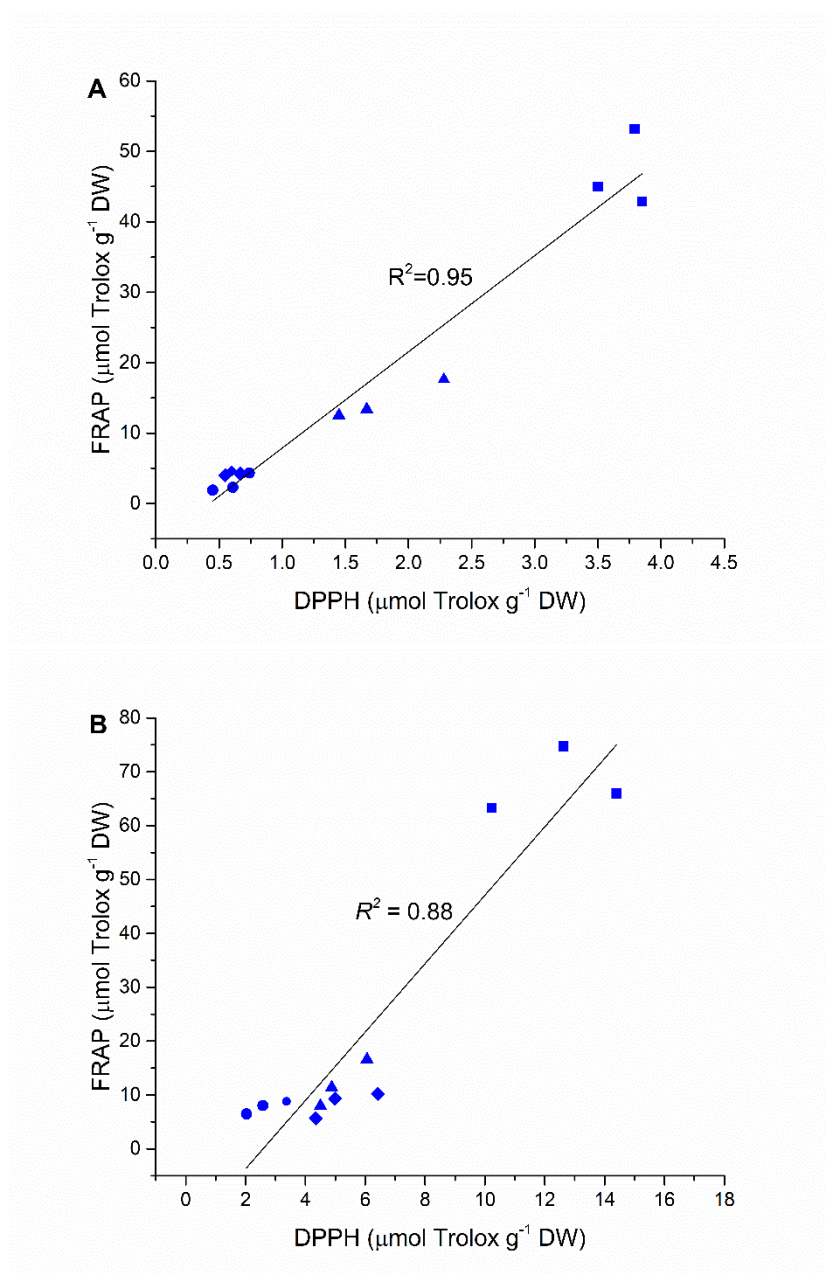
Figure 4.1 Relationship between DPPH and FRAP assays for (A) *Scenedesmus* sp. ME02 and (B) *Hindakia tetrachotoma* ME03. Results of both assays were expressed as micromoles of Trolox equivalent per gram dry weight. The black line represents the regression line. Squares, triangles, circles and diamonds symbolize ethanol/water, ethyl acetate, hexane and water extracts, respectively.

In a previous study, Goiris et al. (2012) determined the antioxidant potential of thirty-two microalgal biomass samples by FRAP and reported a wide range of results from 3.5 to 89.7 µmol TE g^{-1} DW of microalgae. One of the samples identified as *Scenedesmus obliquus* contained 19.7 µmol TE g^{-1} DW, which is two-fold less than the amount measured for *Scenedesmus* sp. ME02 (47.01 ± 3.14 µmol TE g^{-1} DW). In comparison to thirty-two microalgal biomass screened by Goiris et al. (2012), *Hindakia tetrachotoma* ME03 displayed higher antioxidant activity (67.98 ± 3.45 µmol TE g^{-1} DW) except for only one strain, *Phaeodactylum tricornutum* (89.70 ± 1.43 µmol TE g^{-1} DW).

4.5.2   Antioxidant capacity of *Micractinium* sp. ME05

Antioxidant capacity of *Micractinium* sp. ME05 extracts in six different solvents under two different growth regimens was measured by DPPH and FRAP assays. The results are given in Table 4.11 and 4.12. For mixotrophic growth, the highest antioxidant capacity was measured in methanol extracts as 7.72 ± 0.95 µmol TE g^{-1} DW and 93.80 ± 6.28 µmol TE g^{-1} DW followed by ethanol extracts as 6.41 ± 1.33 µmol TE g^{-1} DW and 79.83 ± 7.56 µmol TE g^{-1} DW by DPPH and FRAP assays, respectively. Similarly, the highest antioxidant capacity in heterotrophically grown samples was recorded in methanol extracts as 6.82 ± 1.31 µmol TE g^{-1} DW and 64.91 ± 4.28 µmol TE g^{-1} DW by DPPH and FRAP assays, respectively. The antioxidant capacities of mixotrophically grown microalgae were higher compared to heterotrophic samples. Particularly, the difference in antioxidant capacities measured by FRAP assay between mixotrophic and heterotrophic growth in methanol, ethanol and acetone extracts was statistically significant. The coefficient of determination (R^2) between DPPH and FRAP assays was calculated as 0.57 and 0.65 for mixotrophic and heterotrophic growth, respectively and was highly significant ($p < 0.001$) for both conditions (Figure 4.2). The low correlation between two methods can be explained by the methodological differences in detection and measurement of the antioxidants (Bulut et al. 2019).

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Table 4.11 Antioxidant capacity of mixotrophically cultivated *Micractinium* sp. ME05 in different solvent extracts determined by DPPH and FRAP assays.

Solvent	(%) DPPH Radical scavenging effect [a]	DPPH (μmol TE g^{-1} DW)	FRAP (μmol TE g^{-1} DW)
Methanol	39.61 ± 4.37	7.72 ± 0.95	93.80 ± 6.28
Ethanol	64.15 ± 5.24	6.41 ± 1.33	79.83 ± 7.56
Acetone	46.48 ± 1.39	4.97 ± 0.45	68.88 ± 2.96
Hexane	30.55 ± 19.09	2.05 ± 0.28	15.70 ± 2.25
Ethyl acetate	54.71 ± 1.42	4.10 ± 0.45	29.06 ± 6.32
Water	17.65 ± 2.91	2.93 ± 0.64	7.69 ± 0.43

Results are mean ± standard error of three measurements (n=3). [a] Radical scavenging effects of algal extracts at 1 mg mL^{-1} concentration.

Table 4.12 Antioxidant capacity of heterotrophically cultivated *Micractinium* sp. ME05 in different solvent extracts determined by DPPH and FRAP assays.

Solvent	(%) DPPH Radical scavenging effect [a]	DPPH (μmol TE g^{-1} DW)	FRAP (μmol TE g^{-1} DW)
Methanol	28.10 ± 2.36	6.82 ± 1.31	64.91 ± 4.28
Ethanol	35.75 ± 3.94	3.82 ± 0.62	50.43 ± 10.45
Acetone	44.35 ± 9.44	2.32 ± 0.30	35.70 ± 1.85
Hexane	18.63 ± 5.11	1.12 ± 0.47	11.73 ± 2.14
Ethyl acetate	31.30 ± 3.42	2.79 ± 0.54	14.55 ± 3.33
Water	9.00 ± 2.01	1.68 ± 0.53	1.40 ± 0.50

Results are mean ± standard error of three measurements (n=3). [a] Radical scavenging effects of algal extracts at 1 mg mL^{-1} concentration.
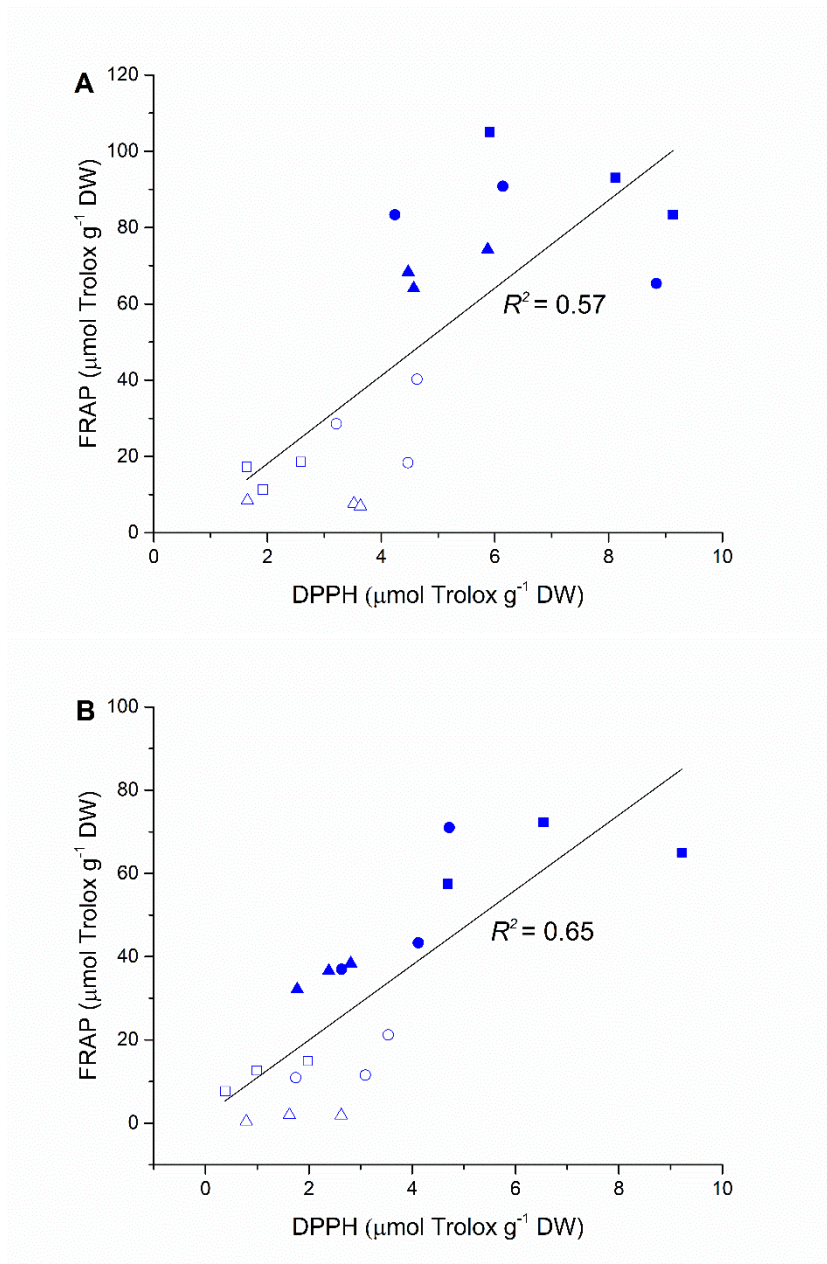
Figure 4.2 Relationship between DPPH and FRAP assays for (A) mixotrophically and (B) heterotrophically cultivated *Micractinium* sp. ME05. Results of both assays were expressed as micromoles of Trolox equivalent per gram dry weight. The black line represents the regression line. Empty squares, filled squares, empty triangles, filled triangles, empty circles and filled circles represent hexane, methanol, water, acetone, ethyl acetate and ethanol extracts, respectively.

The antioxidant capacities of various macro- and microalgae have been determined to date (Li et al. 2007; Goiris et al. 2012; Farvin and Jacobsen, 2013; Machu et al. 2015). To our knowledge, this is the first study that evaluates the antioxidant capacity of a *Micractinium* species. It is a challenge to compare the antioxidant capacities of microalgal strains evaluated in different studies due to principle differences in laboratory conditions and methodology. The antioxidant capacity of *Micractinium* sp. ME05 was higher than that of *Scenedesmus* sp. ME02 in ethyl acetate, hexane and water solvent that are in common for both studies-extracts of mixotrophically grown cultures as measured by DPPH and FRAP assays. Whereas, the antioxidant capacity of *Hindakia tetrachotoma* ME03 in the same solvent extracts was higher than that of both *Scenedesmus* sp. ME02 and *Micractinium* sp. ME05; differences between extracts of *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 were not statistically significant as measured by DPPH and FRAP assays.

Heterotrophic cultivation of *Micractinium* sp. ME05 is advantageous due to higher biomass obtained similar to other microalgae and lower cost since molasses and vinasse, by-products of sugar refinery can effectively be used as the sole carbon source (Engin et al. 2018a; Engin et al. 2018b; Engin et al. 2018c). There is limited information on the effect of heterotrophic growth on antioxidant capacity of microalgae in comparison to other cultivation conditions. In a previous study, Shetty and Sibi compared the antioxidant activities of two microalgae, *Chlorella vulgaris* and *Scenedesmus obliquus*, under autotrophic, mixotrophic and heterotrophic conditions (Shetty and Sibi, 2015). In accordance with the present study, methanol extracts of both microalgae showed lower antioxidant potential when grown heterotrophically compared to autotrophic and mixotrophic growth. Environmental factors such as light and ultraviolet exposure as well as internal processes such as photosynthesis generate ROS and antioxidants are produced to counteract the oxidative damage as a defense mechanism. Microalgae grown under mixotrophic cultivation are exposed to light and rely on both photosynthesis and an additional carbon source provided in the culture medium for energy. Hence, the antioxidant

capacity is likely to be higher in mixotrophic cultures compared to heterotrophic growth conditions due to increased antioxidant activity in response to photosynthesis and light exposure (Zhan et al. 2017).

4.6 Correlation of antioxidant capacity with phenolic, flavonoid, and carotenoid content

Different factors may collectively contribute to the antioxidant capacity of plants or microalgae. Therefore, the coefficient of correlation (r) values between the antioxidant capacity and the total phenolic, flavonoid and carotenoid contents of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 were separately assessed in different solvents.

In the case of *Scenedesmus* sp. ME02, statistical analysis showed that the coefficient of correlation between the FRAP assay and total phenolics extracted in ethanol/water was significant ($p < 0.05$). This is the only statistically significant correlation determined based on the data. Although the coefficients of correlation between total phenolic content in ethyl acetate and both DPPH ($r = 0.99$) and FRAP ($r = 0.98$) assays was high, results were not statistically significant ($p > 0.05$) (Table 4.13).

Strikingly, the coefficient of correlation values between the FRAP assay and total phenolics in ethanol/water ($r = 0.89$), ethyl acetate ($r = 0.93$) and hexane ($r = 0.92$) were considerably high in *Hindakia tetrachotoma* ME03. In addition, the coefficient of correlation between total phenolic content and DPPH assay in hexane extract was 0.99. However, none of the results were statistically significant ($p > 0.05$) (Table 4.14). In contrast to *Scenedesmus* sp. ME02, the coefficient of correlation values between total carotenoid and antioxidant capacity (in particular FRAP assay) was very high in all solvent extracts. But these results were not statistically significant, either ($p > 0.05$).

Table 4.13 The coefficient of correlation values for relation between total phenolic content, total flavonoid content, and carotenoid content and antioxidant capacity in different solvent extracts of *Scenedesmus* sp. ME02.

	Solvent	DPPH	FRAP
Total Phenolic Content	Ethanol/water	$r = 0.14$; p value = 0.91	$r = 0.99$; p value = 0.02
	Ethyl acetate	$r = 0.99$; p value = 0.05	$r = 0.98$; p value = 0.11
	Hexane	$r = 0.37$; p value = 0.76	$r = 0.14$; p value = 0.97
	Water	$r = 0.95$; p value = 0.20	$r = 0.10$; p value = 0.96
Total Flavonoid Content	Ethanol/water	$r = 0.99$; p value = 0.07	$r = 0.28$; p value = 0.82
	Ethyl acetate	$r = 0.50$; p value = 0.66	$r = 0.41$; p value = 0.73
Total Carotenoid Content	Ethanol/water	$r = 0.72$; p value = 0.48	$r = 0.56$; p value = 0.62
	Ethyl acetate	$r = 0.98$; p value = 0.11	$r = 0.96$; p value = 0.17
	Hexane	$r = 0.95$; p value = 0.19	$r = 0.74$; p value = 0.47

Table 4.14 The coefficient of correlation values for relation between total phenolic content, total flavonoid content, and carotenoid content and antioxidant capacity in different solvent extracts of *Hindakia tetrachotoma* ME03.

	Solvent	DPPH	FRAP
Total Phenolic Content	Ethanol/water	$r = 0.70$; p value = 0.50	$r = 0.89$; p value = 0.29
	Ethyl acetate	$r = 0.86$; p value = 0.34	$r = 0.93$; p value = 0.23
	Hexane	$r = 0.99$; p value = 0.07	$r = 0.92$; p value = 0.26
	Water	$r = 0.41$; p value = 0.72	$r = 0.83$; p value = 0.37
Total Flavonoid Content	Ethanol/water	$r = 0.50$; p value = 0.66	$r = 0.97$; p value = 0.13
	Ethyl acetate	$r = 0.82$; p value = 0.38	$r = 0.71$; p value = 0.49
	Hexane	$r = 0.79$; p value = 0.42	$r = 0.57$; p value = 0.61
	Water	$r = 0.61$; p value = 0.58	$r = 0.93$; p value = 0.22
Total Carotenoid Content	Ethanol/water	$r = 0.68$; p value = 0.52	$r = 0.91$; p value = 0.27
	Ethyl acetate	$r = 0.97$; p value = 0.04	$r = 0.99$; p value = 0.06
	Hexane	$r = 0.99$; p value = 0.11	$r = 0.89$; p value = 0.30
	Water	$r = 0.62$; p value = 0.57	$r = 0.94$; p value = 0.21

The coefficient of correlation values between the antioxidant capacity and total phenolics, flavonoids and carotenoids of *Micractinium* sp. ME05 in different solvent extracts were calculated both for mixotrophic and heterotrophic growth conditions (Table 4.15 and 4.16).

Table 4.15 The coefficient of correlation values for relation between total phenolic content or total flavonoid content and antioxidant capacity in various solvent extracts of *Micractinium* sp. ME05 grown under the mixotrophic conditions.

	Solvent	DPPH	FRAP
Total Phenolic Content	Methanol	$r = 0.36$; p value = 0.77	$r = 0.20$; p value = 0.86
	Ethanol	$r = 0.99$; p value = 0.01	$r = 0.77$; p value = 0.44
	Acetone	$r = 0.56$; p value = 0.63	$r = 0.10$; p value = 0.94
	Hexane	$r = 0.98$; p value = 0.16	$r = 0.61$; p value = 0.58
	Ethyl acetate	$r = 0.98$; p value = 0.12	$r = 0.32$; p value = 0.79
	Water	$r = 0.48$; p value = 0.68	$r = 0.10$; p value = 0.95
Total Flavonoid Content	Methanol	$r = 0.28$; p value = 0.82	$r = 0.14$; p value = 0.92
	Ethanol	$r = 0.89$; p value = 0.29	$r = 0.97$; p value = 0.16
	Acetone	$r = 0.95$; p value = 0.19	$r = 0.98$; p value = 0.12
	Hexane	$r = 0.88$; p value = 0.31	$r = 0.10$; p value = 0.95
	Ethyl acetate	$r = 0.63$; p value = 0.56	$r = 0.68$; p value = 0.53
	Water	$r = 0.97$; p value = 0.16	$r = 0.99$; p value = 0.10
Total Carotenoid Content	Methanol	$r = 0.69$; p value = 0.52	$r = 0.57$; p value = 0.61
	Acetone	$r = 0.90$; p value = 0.28	$r = 0.60$; p value = 0.59
	Ethyl acetate	$r = 0.91$; p value = 0.26	$r = 0.53$; p value = 0.64

The coefficient of correlation value between the DPPH assay and total phenolics in ethanol extracts of mixotrophically cultivated microalgae was 0.99 ($p = 0.01$). This result is consistent with the strong correlation between total phenolic content of *Chlorella vulgaris* and *Scenedesmus obliquus* and the DPPH assay reported in Shetty and Sibi (2015). In the same study, it was shown that the contribution of phenolics to the antioxidant potential was irrespective of the cultivation mode (Shetty and Sibi, 2015). In present study, other coefficient of correlation values of 0.90 or higher were

obtained between DPPH or FRAP assay and total phenolic, total flavonoid and total carotenoid contents in various solvent extracts of *Micractinium* sp. ME05 both under mixotrophic and heterotrophic growth conditions; however, none of them were found to be statistically significant ($p > 0.05$).

Table 4.16 The coefficient of correlation values for relation between total phenolic content or total flavonoid content and antioxidant capacity in various solvent extracts of *Micractinium* sp. ME05 grown under the heterotrophic conditions.

	Solvent	DPPH	FRAP
Total Phenolic Content	Methanol	$r = 0.26$; p value $= 0.83$	$r = 0.99$; p value $= 0.10$
	Ethanol	$r = 0.78$; p value $= 0.43$	$r = 0.99$; p value $= 0.06$
	Acetone	$r = 0.49$; p value $= 0.67$	$r = 0.37$; p value $= 0.75$
	Hexane	$r = 0.62$; p value $= 0.57$	$r = 0.85$; p value $= 0.35$
	Ethyl acetate	$r = 0.62$; p value $= 0.57$	$r = 0.98$; p value $= 0.09$
	Water	$r = 0.78$; p value $= 0.43$	$r = 0.26$; p value $= 0.82$
Total Flavonoid Content	Methanol	$r = 0.84$; p value $= 0.37$	$r = 0.84$; p value $= 0.36$
	Ethanol	$r = 0.89$; p value $= 0.30$	$r = 0.99$; p value $= 0.08$
	Acetone	$r = 0.79$; p value $= 0.42$	$r = 0.86$; p value $= 0.34$
	Hexane	$r = 0.10$; p value $= 0.98$	$r = 0.37$; p value $= 0.76$
	Ethyl acetate	$r = 0.68$; p value $= 0.53$	$r = 0.99$; p value $= 0.05$
	Water	$r = 0.10$; p value $= 0.95$	$r = 0.65$; p value $= 0.55$
Total Carotenoid Content	Methanol	$r = 0.81$; p value $= 0.40$	$r = 0.87$; p value $= 0.32$
	Acetone	$r = 0.35$; p value $= 0.77$	$r = 0.22$; p value $= 0.85$
	Ethyl acetate	$r = 0.14$; p value $= 0.90$	$r = 0.78$; p value $= 0.42$

Both Ferric Reducing Antioxidant Power (FRAP) assay and Folin-Ciocalteu method for determination of total phenolics rely on the same principle of metal reduction; whereas DPPH assay relies on the radical scavenging activity for assessment of antioxidant capacity. This principal difference may explain why total phenolics in ethanol/water are a significant contributor to antioxidant potential as determined by FRAP but not the DPPH assay (Hajimahmoodi et al. 2010). In a study by Li et al. (2007) the correlation between phenolic content and antioxidant capacity of 23

microalgae was not found to be significant. In another study, Goiris et al. (2012) determined that phenolic content significantly correlated with Trolox Equivalent Antioxidant Capacity (TEAC) and FRAP assays for thirty-two microalgal biomass samples studied. Carotenoid content was also assessed as a significant contributor to antioxidant capacity. In present study, the coefficient of correlation between carotenoid content and both DPPH and FRAP assays were high ($r = 0.98$ and $r = 0.96$, respectively) but the results were not statistically significant ($p > 0.05$). Shetty and Sibi (2015) reported that the coefficient of correlation between phenolic content and DPPH assay of a *Scenedesmus* strain was statistically significant. Taken together, there is no established evidence on the general relation between total phenolic, flavonoid and carotenoid content of microalgae and their antioxidant capacity in literature. Despite the well-established role of phenolic compounds in relation to the antioxidant capacity of plants, the relative contribution of phenolics to antioxidant potential of microalgae is still under debate (Li et al. 2007; Shetty and Sibi, 2015; Bulut et al. 2019). Other factors such as the fatty acid composition (high levels of PUFAs) may contribute to the antioxidant capacity of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 (Custódio et al. 2014; Aremu et al. 2016; Sonmez et al. 2016).

4.7   RP-HPLC analysis of selected phenolic compounds in microalgal extracts

In this study, amounts of twelve different phenolic compounds that fall in three main categories; namely, benzoic acid derivatives; gallic acid, benzoic acid, 4-hydroxy benzoic acid, vanillic acid, and syringic acid; cinnamic acid and derivatives coumaric acid, caffeic acid, chlorogenic acid, and rosmarinic acid; and flavonols; quercetin and rutin were quantified by Reversed-Phase high-performance liquid chromatography (RP-HPLC) method. Ethanol/water and ethyl acetate extracts of *Scenedesmus* sp. ME02 (Table 4.17) and *Hindakia tetrachotoma* ME03 (Table 4.18), and methanol, acetone and ethyl acetate extracts of mixotrophically and

heterotrophically grown *Micractinium* sp. ME05 (Table 4.19) were used in the chromatographic analyses.

4.7.1 RP-HPLC analysis of selected phenolic compounds in *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03

In *Scenedesmus* sp. ME02, the flavonol, quercetin comprised the highest amount of phenolic compound among the twelve analyzed in the ethyl acetate extract (844.5 ± 125.0 µg g^{-1} DW). Quercetin concentration in ethanol/water extract was also high (551.9 ± 90.9 µg g^{-1} DW). Gallic acid concentration was measured as 653.6 ± 54.3 µg g^{-1} DW in ethanol/water extract and detected to be much lower in the ethyl acetate extract (2.3 ± 0.1 µg g^{-1} DW). Other phenolic compounds such as 4-hydroxy benzoic acid, vanillic acid, caffeic acid and chlorogenic acid were also noticeably higher in the ethanol/water mixture compared to the ethyl acetate extracts (Table 4.17). On the other hand, rosmarinic acid and rutin concentrations were measured as 35.5 ± 2.5 µg g^{-1} DW and 105.8 ± 8.6 µg g^{-1} DW, respectively in the ethyl acetate extract and not detected in ethanol/water mixture.

In *Hindakia tetrachotoma* ME03, quercetin constituted the highest amount of phenolic compound in ethanol/water extract (114.99 ± 8.31 µg g^{-1} DW), which was followed by rutin (77.01 ± 2.84 µg g^{-1} DW) in the same extract. Benzoic acid concentration in ethanol/water extract was also high and measured as 23.80 ± 10.88 µg g^{-1} DW. Strikingly, the amount of benzoic acid was recorded as 42.72 ± 23.33 2.3 ± 0.1 µg g^{-1} DW. The amounts of other phenolic compounds including gallic acid, vanillic acid, syringic acid, coumaric acid, chlorogenic acid and rosmarinic acid were relatively higher in ethanol/water extract compared to the ethyl acetate extracts. In addition, concentration of 4-hydroxy benzoic acid, cinnamic acid and caffeic acid were found to be 15.29 ± 0.50 µg g^{-1} DW, 2.35 ± 1.31 µg g^{-1} DW and 1.70 ± 0.23 µg g^{-1} DW in ethanol/water extract, respectively; whereas same phenolic compounds were not detected in ethyl acetate extract (Table 4.18).

Table 4.17 Phenolic compounds extracted in ethanol/water and ethyl acetate identified by RP-HPLC analysis in *Scenedesmus* sp. ME02.

Phenolic Compound	Amount[a] (μg g^{-1} dry weight)	
	ethanol/water	ethyl acetate
Benzoic acid derivatives		
Gallic acid	653.6 ± 54.3	2.3 ± 0.1
Benzoic acid	ND[b]	14.7 ± 3.6
4-Hydroxy Benzoic acid	441.9 ± 30.0	10.3 ± 3.3
Vanillic acid	83.2 ± 7.3	ND
Syringic acid	ND	4.5 ± 2.2
Cinnamic acid derivatives		
Cinnamic acid	ND	4.0 ± 0.7
Coumaric acid	ND	4.9 ± 0.9
Caffeic acid	67.6 ± 24.7	1.4 ± 0.3
Chlorogenic acid	352.0 ± 45.0	7.2 ± 0.2
Rosmarinic acid	ND	35.5 ± 2.5
Flavonols		
Quercetin	551.9 ± 90.9	844.5 ± 125.0
Rutin	ND	105.8 ± 8.6

[a] Results are mean ± standard error of two measurements.
[b] Not detected.

Table 4.18 Phenolic compounds extracted in ethanol/water and ethyl acetate identified by RP-HPLC analysis in *Hindakia tetrachotoma* ME03.

Phenolic Compound	Amount[a] ($\mu g\ g^{-1}$ dry weight)	
	ethanol/water	ethyl acetate
Benzoic acid derivatives		
Gallic acid	26.11 ± 0.15	2.14 ± 0.15
Benzoic acid	23.80 ± 10.88	42.72 ± 23.33
4-Hydroxy Benzoic acid	15.29 ± 0.50	ND[b]
Vanillic acid	2.32 ± 0.70	0.48 ± 0.30
Syringic acid	13.47 ± 5.39	2.75 ± 0.03
Cinnamic acid derivatives		
Cinnamic acid	2.35 ± 1.31	ND[b]
Coumaric acid	8.78 ± 1.01	1.26 ± 0.06
Caffeic acid	1.70 ± 0.23	ND[b]
Chlorogenic acid	1.42 ± 0.17	0.33 ± 0.04
Rosmarinic acid	8.16 ± 0.87	2.86 ± 0.66
Flavonols		
Quercetin	114.99 ± 8.31	1.39 ± 0.14
Rutin	77.01 ± 2.84	ND[b]

[a] Results are mean ± standard error of two measurements.
[b] Not detected.

There are few studies on detection of phenolics in microalgae by HPLC to compare these results and to the best of our knowledge, this is the first study to report the amounts of selected phenolic compounds in *Scenedesmus* and *Hindakia* strains. In a previous study by Onofrejová et al. (2010) the freshwater green microalga *Spongiochloris spongiosa* contained lower amounts of phenolic compounds, namely; chlorogenic acid, caffeic acid, coumaric acid and cinnamic acid compared to *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03 analyzed in current study. Yet in another study by Machu et al. (2015), *Chlorella pyrenoidosa* was shown to contain 5 $\mu g\ g^{-1}$ DW gallic acid and 20 $\mu g\ g^{-1}$ DW 4-hydroxy benzoic acid. Gallic

acid (653.6 µg g^{-1} DW and 26.11 µg g^{-1} DW) and 4-hydroxy benzoic acid (441.9 µg g^{-1} DW and 15.29 µg g^{-1} DW) amounts were much higher in ethanol/water extracts of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03, respectively.

Compared to microalgae, macroalgae (i.e. seaweeds) have gained considerable attention in terms of their antioxidant potential and phenolic compounds. In a study by López et al. (2011), gallic acid was found to be the predominant polyphenol in brown alga *Stypocaulon scoparium*, and its amount highly varied in different solvent extracts; whereas quercetin and rutin were significantly lower than the amounts in the present study. Quercetin is a flavonoid that is found in high amounts in vegetables and fruit such as caper, onion, cranberry and apples. It has a wide range of health benefits including anti-obesity, anti-carcinogenic, anti-inflammatory and antibacterial effects (Wang et al. 2016). Quercetin content of *Scenedesmus* sp. ME02 ($\sim$80 mg 100 g^{-1} DW in ethyl acetate extracts) is comparable to the quercetin levels in a selected list of vegetables, fruit and beverages. Rutin, on the other hand, is a quercetin derivative and is found abundantly in cherry and spinach (Wang et al. 2016) but has been previously reported in one study with diatom *Phaeodactylum tricornutum* (Rico et al. 2013). Although different quantification methods were used, our data indicate that quercetin and rutin predominantly account for the total flavonoid content in our samples. To the best of our knowledge, this is the first study that establishes the presence of flavonoids, rutin and quercetin in high amounts in green microalgal species.

Finally, despite *Hindakia tetrachotoma* ME03 had higher total phenolic and flavonoid contents compared to those of *Scenedesmus* sp. ME02 in both ethanol/water and ethyl acetate extracts, amounts of individual phenolic compounds were significantly lower. This might be caused by the presence of other phenolic compounds that have not been included in this study at much higher concentrations in *Hindakia tetrachotoma* ME03.

4.7.2 RP-HPLC analysis of selected phenolic compounds in *Micractinium* sp. ME05

The above-mentioned twelve phenolic compounds were also quantified by RP-HPLC method in methanol, acetone and ethyl acetate extracts of mixotrophically and heterotrophically grown *Micractinium* sp. ME05 (Table 4.19). Gallic acid (469.21 ± 159.74 µg g^{-1} DW) in acetone extracts of mixotrophic microalgae was the highest phenolic compound detected. Under heterotrophic growth, 4-hydroxy benzoic acid (403.93 ± 20.98 µg g^{-1} DW) in methanol extracts was the highest phenolic compound. Strikingly, the amount of the same compound in methanol extracts of mixotrophic *Micractinium* sp. ME05 was only 1.98 ± 0.91 µg g^{-1} DW. Acetone is a powerful solvent of flavonols and consistently rutin concentration in acetone extracts (212.09 ± 122.46 µg g^{-1} DW in mixotrophic samples) was significantly higher than other solvents. Overall, there were significant differences in the amounts of phenolic compounds between mixotrophic and heterotrophic microalgal extracts. The choice of solvent for extraction also affected the amount of the specific phenolic compound recovered.

To our knowledge, this is the first study that quantifies the above-mentioned phenolic compounds in a *Micractinium* species. It is also the first time that a study compares the relative quantities of specific phenolic compounds under two cultivation modes in microalgae. Another freshwater green microalga, *Chlorella pyrenoidosa* had considerably lower (5 µg g^{-1} sample) gallic acid concentration than *Micractinium* sp. ME05 (Machu et al. 2015). 4-hydroxy benzoic acid content (20 µg g^{-1} sample) was higher in *C. pyrenoidosa* compared to mixotrophic *Micractinium* sp. (~2 µg g^{-1} DW in methanol extract) but much lower than heterotrophically grown culture (~400 µg g^{-1} DW in methanol extract). The growth conditions of *C. pyrenoidosa* were not specified. 4-hydroxy benzoic acid is known for its antimicrobial activity and is used as a preservative in a wide range of industries including food, pharmaceutical and cosmetics. It is yet unclear why 4-hydroxy benzoic acid content increased two hundred times under heterotrophic growth than mixotropic growth.

Table 4.19 Phenolic compounds in the different solvent extracts of *Micractinium* sp. ME05 identified by RP-HPLC analysis.

Phenolic compound	Amount[a] (μg g^{-1} DW)					
	Mixotrophic growth			Heterotrophic growth		
	Methanol	Acetone	Ethyl acetate	Methanol	Acetone	Ethyl acetate
Benzoic acid derivatives						
Gallic acid	129.08 ± 2.65	469.21 ± 159.74	ND[b]	125.06 ± 11.68	12.93 ± 3.93	ND
Benzoic acid	13.62 ± 2.63	37.84 ± 2.20	8.77 ± 2.55	107.20 ± 16.85	28.09 ± 14.36	18.50 ± 3.56
4-Hydroxy Benzoic acid	1.98 ± 0.91	0.95 ± 0.12	1.10 ± 0.63	403.93 ± 20.98	40.48 ± 4.10	4.75 ± 0.60
Vanillic acid	13.37 ± 7.72	5.37 ± 1.54	ND	47.66 ± 2.44	11.91 ± 1.19	4.46 ± 0.18
Syringic acid	27.99 ± 6.87	9.32 ± 0.58	5.01 ± 0.61	56.42 ± 13.04	5.70 ± 0.53	6.77 ± 0.88
Cinnamic acid derivatives						
Cinnamic acid	10.34 ± 6.86	18.06 ± 0.77	ND	196.44 ± 6.70	51.88 ± 2.26	38.24 ± 3.61
Coumaric acid	19.36 ± 15.25	4.41 ± 1.46	9.58 ± 1.29	10.85 ± 1.49	0.55 ± 0.43	5.94 ± 0.02
Caffeic acid	16.46 ± 9.50	4.13 ± 0.99	ND	3.87 ± 0.86	4.51 ± 0.30	ND
Chlorogenic acid	11.27 ± 5.61	2.30 ± 0.10	1.55 ± 0.89	2.78 ± 1.05	0.79 ± 0.55	1.86 ± 0.08
Rosmarinic acid	34.84 ± 2.89	1.83 ± 0.51	1.98 ± 1.15	18.45 ± 10.60	ND	4.22 ± 0.59
Flavonols						
Quercetin	65.63 ± 0.49	2.70 ± 0.94	30.02 ± 13.28	37.09 ± 8.67	28.20 ± 5.81	14.24 ± 1.66
Rutin	53.91 ± 0.58	212.09 ± 122.46	ND	39.89 ± 0.10	80.33 ± 38.33	ND

[a] Results are mean ± standard error of two measurements. [b] Not detected

Interestingly, although *Micractinium* sp. ME05 had higher total flavonoid content compared to that of *Scenedesmus* sp. ME02, quercetin and rutin concentrations were significantly lower. It can, thus be speculated that other flavonoids that have not been included in this study are more abundant in *Micractinium* sp. ME05 (Bulut et al. 2019). Cinnamic acid derivatives, caffeic acid and chlorogenic acid are also considerably higher in *Scenedesmus* sp. ME02 compared to *Micractinium* sp. ME02.

Goiris et al. (2014) demonstrated that although all major evolutionary lineages of microalgae had the capacity to produce flavonoids, the relative amounts of individual compounds were highly variable among different samples. These data further suggest that the flavonoid contents are also variable in three thermo-tolerant freshwater green microalgae collected from the same geographical location.

4.8 Cytotoxicity of microalgal extracts

The cytotoxic effects of ethanol/water extracts of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03, and methanol extracts of mixotrophically cultivated *Micractinium* sp. ME05 were assessed against HeLa and MCF-7 cells by MTT assay. The working principle of MTT assay is based on the reduction of the yellow aqueous solution of tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl) to a violet blue/purple colored water insoluble dye compound, formazan by mitochondrial dehydrogenases present in metabolically active cells. Hence, the amount of formazan is directly proportional to the number of viable cells (Mosmann, 1983).

The viability of control cells treated with the vehicle solution (methanol) was accepted as 100% and the relative viability of the cells treated with various concentrations of the microalgal extracts was calculated accordingly. Hydrogen peroxide at 0.5 mM concentration was also used as a positive control. According to the data obtained by MTT assay, extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 did not show any cytotoxic effect on HeLa and MCF-7 cells at all concentrations tested. As shown in Figure 4.3, the cell viability of HeLa and MCF-7 cells was within 93.73-99.51% and 91.46-98.85%,

respectively when treated with microalgal extracts at concentrations of 12.5-400 µg mL^{-1}. Further, the viability of both HeLa and MCF-7 cells treated with various concentrations of extracts was not significantly different as compared with the control group ($p > 0.05$). However, hydrogen peroxide decreased the viability of HeLa and MCF-7 cells to 32.54% and 35.43%, respectively. These results show that the extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 are non-toxic at any concentrations.

The cytotoxic and anti-proliferative effects of plant or algae derived extracts have been extensively studied. Previously, Nigjeh et al. (2013) reported the dose-dependent anti-proliferative effects of ethanolic extracts of a microalga, *Chaetoceros calcitran* on MCF-7 cells. The IC50 value of ethanol extract on MCF-7 cells was determined to be 3.00 ± 0.65 µg mL^{-1} for 24-hour exposure, which was further reduced to 2.69 ± 0.24 µg mL^{-1} after treatment for 72 h. Similarly, Suh et al. (2017) showed the cytotoxic effects of extracts of freshwater microalga, *Chloromonas* sp. on various cell lines in a dose-dependent manner, which was attributed to the antioxidant capacity of the extracts. Interestingly, same extracts did not exert any anti-proliferative effect on another cell line. In contrast, there have been various studies indicating the non-toxicity of algal extracts against various cell lines in spite of their high antioxidant capacity (Kang et al. 2015; Pinteus et al. 2017). For instance, Sansone et al. (2017) showed that ethanol/water extract of the marine green microalga, *Tetraselmis suecica* containing high levels of carotenoids had a significant antioxidant capacity and induced the cell repairing through up-regulation of expression of the genes involved in oxidative stress and repairing pathways. Since many studies reporting different results regarding the toxicity and anti-proliferative activity, it is difficult to make a certain conclusion about the toxic effects of algal extracts. These differences most probably arise from the differences in algal strains and constituents of algal extracts. Ethanol/water extracts of *Scenedesmus* sp. ME02 and *H. tetrachotoma* ME03, and methanol extracts of *Micractinium* sp. ME05 used in present study did not exhibit any toxicity even at the highest concentration. Thus, the following experiments were conducted within these non-toxic concentrations.
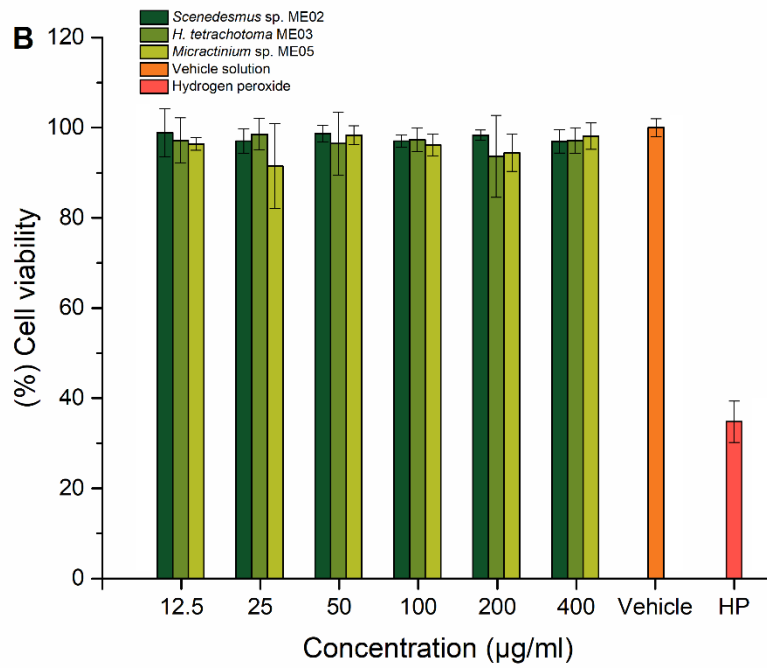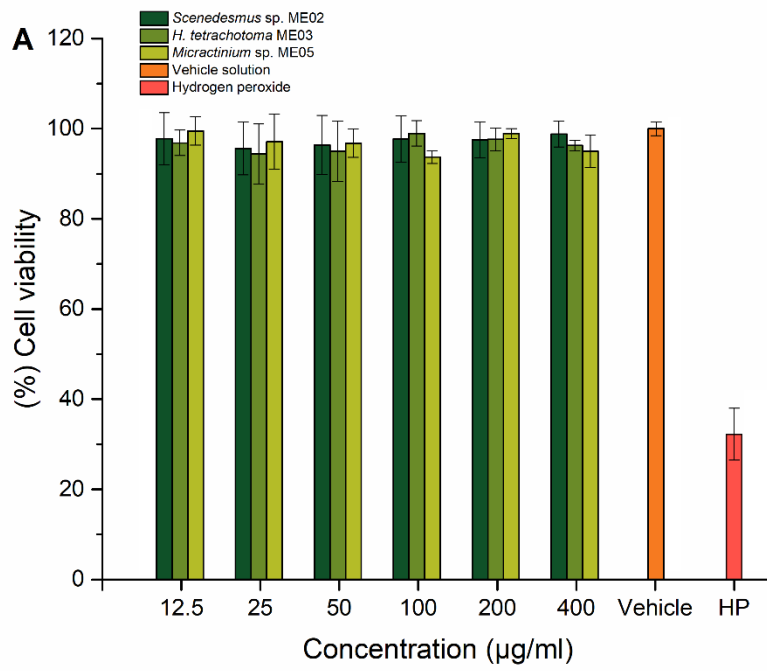
94

Figure 4.3 The viability of (A) HeLa and (B) MCF-7 cells after 48 h incubation with various concentrations of microalgal extracts.

4.9 Inhibitory effects of microalgal extracts on intracellular ROS generation

ROS are free radicals that are generally produced in the mitochondria by the reactions of oxidative phosphorylation. Because of the deleterious effects of ROS, production and scavenging of these molecules are in a dynamic equilibrium in order to main biological functions. However, the impaired balance between ROS production and removal results in oxidative stress which is attributed to different types of chronic diseases and pathological conditions by causing protein and lipid oxidation, cellular injury and even apoptosis (Droge, 2002).

Hydrogen peroxide (H_2O_2) is the most common ROS generator and can cause the accumulation of intracellular ROS at elevated concentrations, thereby leading to cell damage by affecting cellular defense mechanisms. Therefore, cell-permeable H_2O_2 has been extensively used in *in vitro* studies as a ROS generator (Zhuang et al. 2017). In present study, ethanol/water extracts of *Scenedesmus* sp. ME02 and *Hindakia tetrachotoma* ME03, and methanol extracts of mixotrophically cultivated *Micractinium* sp. ME05 were investigated for their intracellular antioxidant activities against H_2O_2-induced ROS production in MCF-7 cells. Since the cellular viability study revealed that none of the extracts displayed toxicity on both HeLa and MCF-7 cells at any concentrations, extracts at 50 μg mL^{-1}, 100 μg mL^{-1}, 200 μg mL^{-1} and 400 μg mL^{-1} were used as the test concentrations in order to evaluate intracellular ROS scavenging ability. After incubation of MCF-7 cells with of microalgal extracts, the cells were exposed to 0.5 mM of H_2O_2 for 6 h. Then, intracellular ROS levels were examined by 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) assay. This method uses the cell-permeable H_2DCFDA that measures hydroxyl, peroxyl and other ROS within the cell. Upon its diffusion into the cell, non-fluorescent DCFH-DA is oxidized by intracellular ROS into 2',7'-dichlorodihydrofluorescein (DCF), which is a highly fluorescent compound and its fluorescence signal is proportional to the amounts of intracellular ROS (Oparka et al. 2016). The results are given in Figure 4.4.

After treatment of MCF-7 cells with H_2O_2, the relative fluorescence intensity of the control group that was treated with only vehicle solution (methanol) was determined to be around 28,000 (in arbitrary unit, AU). However, *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 extracts significantly inhibited the generation of intracellular ROS in a concentration-dependent manner ($p < 0.001$). At the highest concentration of 400 μg mL^{-1}, treatment with *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 extracts reduced the production of intracellular ROS in MCF-7 cells by 66.82%, 76.10% and 72.62% compared to the control group, respectively. The differences between inhibitory effects of the microalgal extracts were not statistically significant ($p > 0.05$) at this concentration. Consistent with the concentration-dependent trend, the production of intracellular ROS was inhibited by 18.03%, 29.19% and 23.79% at the lowest concentration of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 extracts, respectively. Only the difference between *Hindakia tetrachotoma* ME03 and *Scenedesmus* sp. ME02 was significant ($p < 0.05$); whereas the rest was not statistically significant ($p > 0.05$).

At each concentration tested, *Hindakia tetrachotoma* ME03 extracts exhibited the strongest scavenging activity of intracellular ROS, which was followed by *Micractinium* sp. ME05 extracts. On the other hand, *Scenedesmus* sp. ME02 extracts showed relatively lower intracellular ROS inhibitory effect in comparison with other extracts, which might be due to differences in the antioxidant capacities, phenolic and flavonoid contents of the extracts.
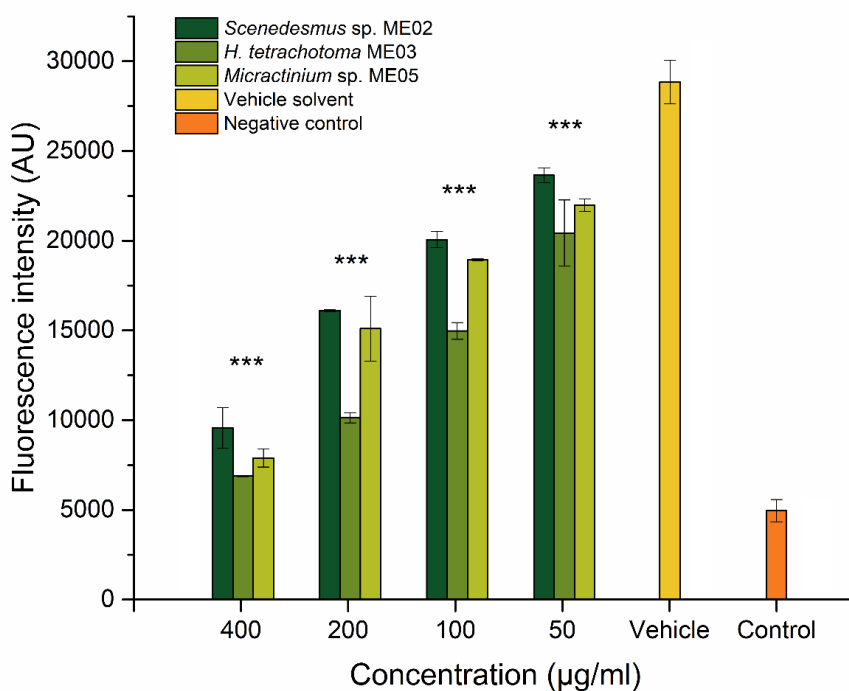
Figure 4.4 Inhibitory effects of the microalgal extracts on intracellular ROS generation in H_2O_2-induced MCF-7 cells. All values are represented as mean ± SD (n =3). Asterisks indicate the significance of the samples with respect to the vehicle solution.

These results show that phenolic extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 possess promising antioxidant effects against H_2O_2-induced oxidative stress in MCF-7 cells. This is consistent with previously studies reported that solvent extracts of various organisms such as plants and algae attenuated the production of intracellular ROS due to their bioactive compound and antioxidant contents (Kang et al. 2015; Heo et al. 2008; Pinteus et al. 2017; Fan et al. 2020). In a previous study, Kang et al. (2015) evaluated the protective effects of methanolic extracts of a brown alga, *Petalonia binghamiae*, against oxidative stress-induced cell damage and reported that the algal extracts inhibited the intracellular ROS production by about 50% when the cells were treated with the extract at the concentration of 300 μg mL^{-1}.

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Similarly, Kang et al. (2004) screened the inhibitory effects of seventeen algal samples on intracellular ROS generation and reported different effects depending on the algal species used. Interstingly, some samples were determined to have no effect on ROS formation and yet some samples increased the levels of intracellular ROS. Only three of algal species, *Ulva pertusa*, *Symphyocladia latiuscula* and *Ecklonia stolonifera* inhibited the ROS generation by 85.65%, 50.63% and 44.30%, respectively. In comparison to seventeen algal biomass screened by Kang et al. (2004), *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 displayed higher inhibitory effect on ROS production except for only one strain, *U. pertusa*.

4.10   Cytoprotective activity against H_2O_2-induced cell apoptosis

Apoptosis is defined as the programmed cell death, which is an important regulatory mechanism of cell growth, development and differentiation. This process plays a vital role in cell homeostasis and differs from the necrotic process which is another form of cell death mediated by irreversible cell injury. However, abnormal apoptosis may cause adverse effects associated with various human conditions such as neurodegenerative disorders, autoimmune diseases, ischemic damage and cancer (Hengartner, 2000; Kannan and Jain, 2000). Since the ability of various compounds to modulate cell homeostasis has a promising therapeutic potential, the cell apoptosis assay is widely used to evaluate the potential and beneficial effects of different types of natural products including bioactive compounds and antioxidants (Cho et al. 2015; Zhuang et al. 2017).

Therefore, Annexin V-FITC/PI apoptosis assay was performed in order to monitor the cell death in MCF-7 cells induced by H_2O_2 and assess whether solvent extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 show cytoprotective activity against H_2O_2-induced cell death at 400 µg mL^{-1} concentration. This assay is based on Annexin V and PI labeling of cells in order to identify cell death and distinguish between its different pathways; apoptosis and

necrosis. Annexin V is a type of intracellular protein that specifically binds to phosphatidylserine (PS) in a calcium-dependent manner. In healthy cells, PS is asymmetrically placed only on inner leaflet of the cell membrane; however during apoptosis, the asymmetry is lost, thus exposing PS to the outer leaflet. Therefore, Annexin V labeled with a fluorescent tag such as FITC can be used to specifically identify apoptotic cells. This assay is usually performed in conjunction with a live/dead dye such as PI in order to distinguish viable cells (Annexin V-FITC negative, PI negative) from early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic cells (AnnexinV-FITC positive, PI positive) and necrotic cells (Annexin V negative, PI positive) (Koopman et al. 1994; Aubry et al. 1999).

As shown in Figure 4.5, when treated with 1 mM H_2O_2, the viability of MCF-7 cells were decreased to $54.83 \pm 3.87\%$, whereas the apoptosis and necrosis rates significantly increased to $10.6 \pm 0.95\%$ and $34.56 \pm 2.92\%$, respectively ($p < 0.001$). As compared with H_2O_2 treatment, the percentage of viability in MCF-7 cells treated with solvent extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 significantly increased ($p < 0.001$). Among all microalgal extracts, *Hindakia tetrachotoma* ME03 displayed the strongest cell protective effect; the apoptosis and necrosis rates were reduced to $5.33 \pm 2.93\%$ and $8.06 \pm 4.93\%$, respectively, which was followed by *Micractinium* sp. ME05 with the apoptosis rate of $8.60 \pm 1.45\%$ and necrosis rate of $17.36 \pm 1.78\%$. *Scenedesmus* sp. ME02 extracts showed the weakest protective effect. Interestingly, the treatment with *Scenedesmus* sp. ME02 extracts resulted in $15.26 \pm 1.65\%$ apoptotic rate, which is higher than H_2O_2 treatment control ($10.6 \pm 0.95\%$). However, *Scenedesmus* sp. ME02 extracts reduced the rate of necrosis from $34.56 \pm 2.92\%$ to $14.90 \pm 1.41\%$. The representative flow cytometry plots of the microalgal extracts and controls are also shown in Figure 4.6.
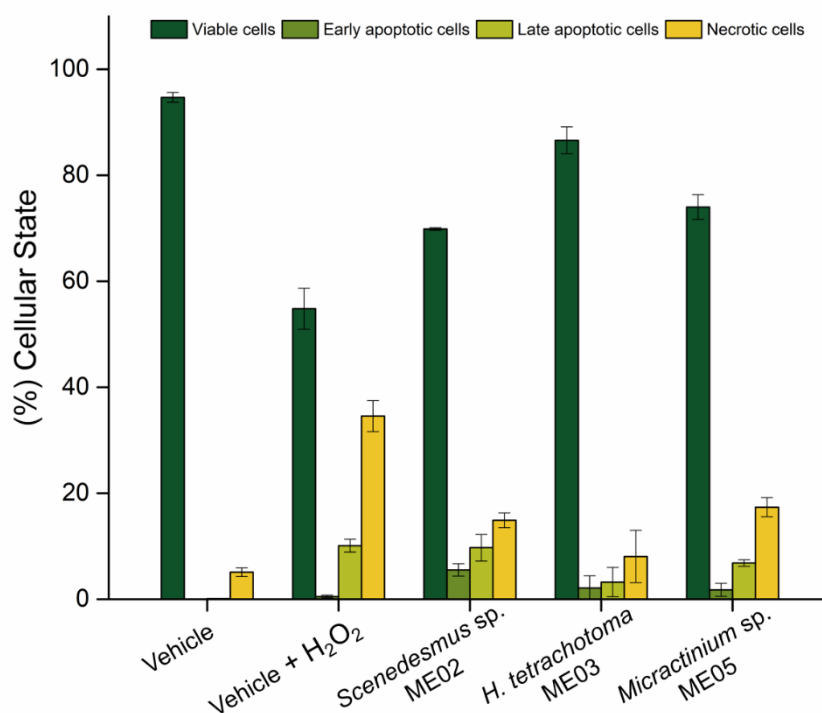
Figure 4.5 Cytoprotective effects of the microalgal extracts in H_2O_2-treated MCF-7 cells. All values are represented as mean ± SD (n =3).

These results show that pretreatment with the extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* sp. ME05 could significantly inhibit H_2O_2-induced cell death in MCF-7 cells by decreasing the apoptotic and necrotic cells ($p < 0.05$). Differences in the effects of microalgal extracts on cell viability, apoptotic and necrotic rates might be due to the content and composition of phenolic compounds and flavonoids. Solvent extracts of various types of plants and algae have been shown to scavenge intracellular ROS and protect cells from oxidative stress-induced cytotoxicity due to their antioxidant capacities (Pinteus et al. 2017; Stranska-Zachariasova et al. 2017; Liu et al. 2019). Consistently, *Hindakia tetrachotoma* ME03 extracts that had the highest total phenolic and flavonoid contents, antioxidant capacities and inhibitory effects on ROS displayed the cytoprotective activity among other microalgal strains.
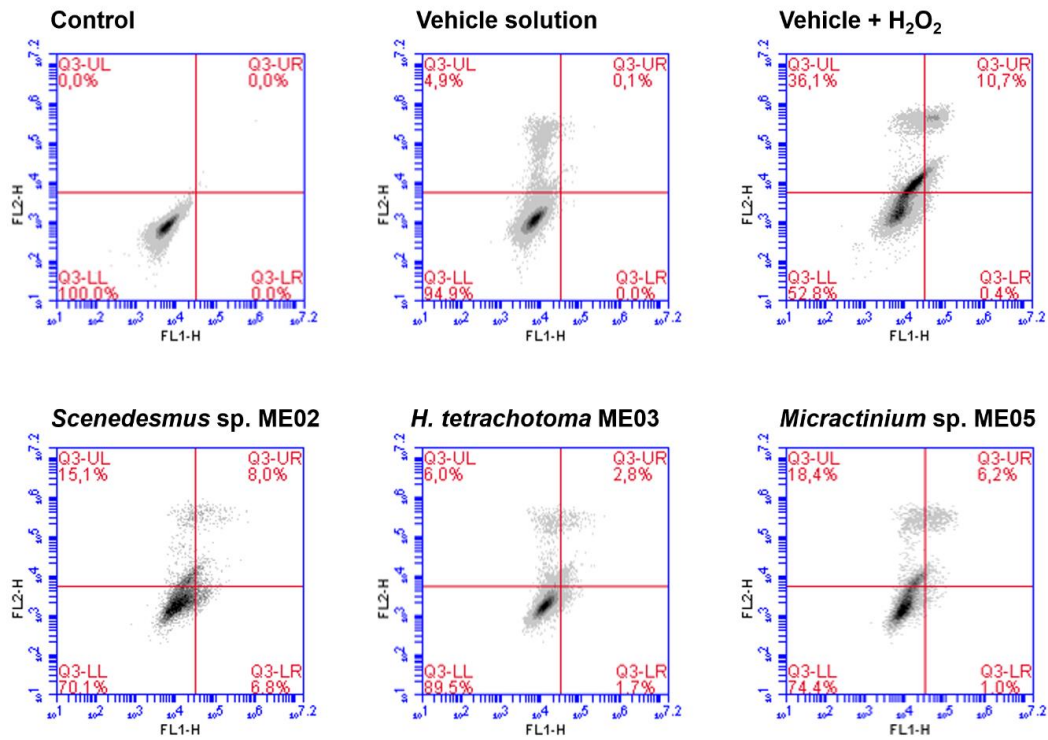
Figure 4.6. Representative flow cytometry plots of Annexin V-FITC/PI apoptosis assay. Lower left quadrant (Annexin V negative, PI negative), lower right qudrant (Annexin V positive, PI negative), upper right qudrant (Annexin V positive, PI positive) and upper left qudrant (Annexin V negative, PI positive) represent viable cells, early apoptotic cells, late apoptotic cells and necrotic cells, respectively.

CHAPTER 5

CONCLUSION

The presence of vast diversity of natural compounds, ease of cultivation and minimum requirement for land are some characteristics of microalgae that may supersede plants for industrial use.

In this study, the potential of three novel green thermo-tolerant microalgae, *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* ME05 was investigated for their use as a natural source of bioactive compounds by determining their antioxidant capacities and the presence of different bioactive compounds including phenolics, flavonoids and carotenoids.

Microalgal strains, *Scenedesmus* sp. ME02 an *Hindakia tetrachotoma* ME03 were cultivated under mixotrophic conditions. *Micractinium* ME05 was grown under two different cultivation modes; mixotrophic and heterotrophic. Heterotrophic cultivation is cost-efficient as continuous external light supply is not necessary. Furthermore, alternative carbon sources such as wastewater or sugarcane molasses can be utilized instead of glucose. Mixotrophic cultivation, on the other hand, is advantageous as the cells can rely on an external carbon source in addition to photosynthesis for growth. *Micractinium* sp. ME05 was previously optimized for heterotrophic growth with success using molasses or vinasse, both of which are byproducts of a sugar factory.

Since the content of extracts depends highly on the type of solvent used, the total phenolic, flavonoid and carotenoid contents and antioxidant capacitites of microalgal species extracted seperately in different solvents with various polarities were compared.

The differences in methodology, solvents used and cultivation conditions hinder direct comparison of our results with previous studies. Nonetheless; overall, microalgal strains used in this study stand out among other microalgae with their high total phenolic contents and antioxidant capacities.

The solvent extracts of *Hindakia tetrachotoma* ME03 were determined to have similar trend according to the nature of the solvent, however total phenolic contents were significantly higher than those of *Scenedesmus* sp. ME02. Similarly, total phenolic contents were determined to be higher in the extracts prepared with polar solvent for both mixotrophically and heterotrophically grown *Micractinium* ME05.

The statistical analysis revealed that the total phenolic content contributed significantly to the antioxidant capacity of microalgal strains. Overall, the extracts of *Hindakia tetrachotoma* ME03 showed higher antioxidant capacity total phenolic, flavonoid and carotenoid contents compared to *Scenedesmus* sp. ME02. In the case of *Micractinium* ME05, mixotrophically grown algal extracts had higher antioxidant activity and bioactive compound contents than heterotrophically grown samples.

Scenedesmus sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* ME05 also showed high amounts of various phenolic compounds including gallic acid, 4-hydroxy benzoic acid, chlorogenic acid, caffeic acid, vanillic acid and quercetin and rutin.

The measurement of total flavonoid content as well as high quercetin and rutin amounts are significant contributions of this study to the present literature on antioxidant activities of microalgae. To our knowledge, this is the first study that reports the amounts of the above-mentioned phenolic compounds in *Hindakia* and *Micractinium* species. Additionally, this is the first time that the antioxidant capacity of a *Micractinium* species is reported and the bioactive compound content of two different cultivation modes has been compared.

Finally, cell culture studies indicated that the solvent extracts of *Scenedesmus* sp. ME02, *Hindakia tetrachotoma* ME03 and *Micractinium* ME05 had no cytotoxic effects on HeLa and MCF-7 cells at any concentration tested. In contrast, the

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microalgal extracts significantly reduced H_2O_2-induced intracellular ROS formation and displayed cytoprotective effects against oxidative stress-induced cytotoxicity due to their antioxidant capacities.

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A. Composition of growth media

Tris-Acetate-Phosphate medium

Table 6.1. Composition of Tris-Acetate-Phosphate medium (Adapted from Culture Collection of Cryophilic Algae).

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
Tris Base	2.42 g	$H_2NC(CH2OH)_3$	-	2.00×10^{-2} M
B-solution	50 mL	NH_4Cl	15.0 g L^{-1}	7.00×10^{-3} M
		$CaCl_2.2H_2O$	4.00 g L^{-1}	8.30×10^{-4} M
		$MgSO_4.2H_2O$	2.00 g L^{-1}	4.50×10^{-4} M
Phosphate Buffer	1 mL	K_2HPO_4	288 g L^{-1}	1.65×10^{-3} M
		KH_2PO_4	144 g L^{-1}	1.05×10^{-3} M
Trace Elements Solution	1 mL	$Na_2EDTA.2H_2O$	50.0 g L^{-1}	1.34×10^{-4} M
		$ZnSO_4.7H_2O$	22.0 g L^{-1}	1.36×10^{-4} M
		H_3BO_3	11.4 g L^{-1}	1.84×10^{-4} M
		$MnCl_2.4H_2O$	5.00 g L^{-1}	4.00×10^{-5} M
		$FeSO_4.7H_2O$	5.00 g L^{-1}	3.29×10^{-5} M
		$CoCl_2.6H_2O$	1.60 g L^{-1}	1.23×10^{-5} M
		$CuSO_4.5H_2O$	1.60 g L^{-1}	1.00×10^{-5} M
		$(NH_4)6MoO_3$	1.10 g L^{-1}	4.44×10^{-6} M
Acetic Acid	1 mL	CH_3COOH	-	-

Ingredients given in Table 6.1 were added to about 800 mL distilled water. After dissolution of ingredients, the final volume was completed to 1 L with distilled water. Final pH was adjusted to to 6.5-7.0, and the medium was sterilized by autoclaving at

121°C for 20 min. For solid medium, 1.5% of agar was added to the medium before autoclaving.

Bold' basal medium

Table 6.2. Composition of Bold's basal medium (Adapted from Culture Collection of Cryophilic Algae).

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
SS 1	10 mL	$NaNO_3$	25.0 g L^{-1}	2.98 x 10^{-3} M
SS 2	10 mL	$MgSO_4.7H_2O$	7.5 g L^{-1}	3.04 x 10^{-4} M
SS 3	10 mL	NaCl	2.5 g L^{-1}	4.28 x 10^{-4} M
SS 4	10 mL	K_2HPO_4	7.5 g L^{-1}	4.31 x 10^{-4} M
SS 5	10 mL	KH_2PO_4	17.5 g L^{-1}	1.29 x 10^{-3} M
SS 6	10 mL	$CaCl_2.2H_2O$	2.5 g L^{-1}	1.70 x 10^{-4} M
SS 7	1 mL	H_3BO_3	11.4 g L^{-1}	1.85 x 10^{-4} M
EDTA-KOH Solution	1 mL	$EDTA.Na_2$	50.0 g L^{-1}	1.71 x 10^{-4} M
		KOH	31.0 g L^{-1}	5.53 x 10^{-4} M
Ferric Solution	1 mL	$FeSO_4.7H_2O$	4.9 g L^{-1}	1.79 x 10^{-5} M
		H_2SO_4	1 mL	-
Trace Elements Solution	1 mL	$ZnSO_4.7H_2O$	8.8 g L^{-1}	3.07 x 10^{-5} M
		$MnCl_2.4H_2O$	1.4 g L^{-1}	7.28 x 10^{-6} M
		MoO_3	0.7 g L^{-1}	4.93 x 10^{-6} M
		$CuSO_4.5H_2O$	1.5 g L^{-1}	6.29 x 10^{-6} M
		$Co(NO_3)2.6H_2O$	0.5 g L^{-1}	1.68 x 10^{-6} M

Solutions given in Table 6.2 were added to about 800 mL distilled water. After dissolution of ingredients, the final volume was completed to 1 L with distilled water. Then, the medium was sterilized by autoclaving at 121°C for 20 min.

BG-11 medium

Table 6.3. Composition of BG-11 medium (Adapted from Culture Collection of Cryophilic Algae).

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
SS 1	60 mL	$NaNO_3$	25.0 g L^{-1}	1.76 x 10^{-2} M
SS 2	10 mL	$MgSO_4.7H_2O$	7.50 g L^{-1}	3.04 x 10^{-4} M
SS 3	20 mL	Na_2CO_3	1.00 g L^{-1}	1.89 x 10^{-4} M
SS 4	5.3 mL	K_2HPO_4	7.50 g L^{-1}	2.28 x 10^{-4} M
SS 5	14 mL	$CaCl_2.2H_2O$	2.50 g L^{-1}	2.38 x 10^{-4} M
SS 6	6 mL	Citric Acid	1.00 g L^{-1}	3.12 x 10^{-5} M
SS 7	10 mL	Ferric Ammonium Citrate	0.60 g L^{-1}	2.26 x 10^{-5} M
SS 8	10 mL	$Na_2EDTA.2H_2O$	0.10 g L^{-1}	2.69 x 10^{-6} M
Trace Elements Solution	1 mL	H_3BO_3	61.0 mg L^{-1}	9.87 x 10^{-7} M
		$MnSO_4.H_2O$	169.0 mg L^{-1}	1.00 x 10^{-6} M
		$ZnSO_4.7H_2O$	287.0 mg L^{-1}	9.98 x 10^{-7} M
		$CuSO_4.5H_2O$	2.5 mg L^{-1}	1.00 x 10^{-8} M
		$(NH_4)6Mo7O_{24}.4H_2O$	12.5 mg L^{-1}	1.01 x 10^{-8} M

Solutions given in Table 6.3 (except for SS 6 and 7) were added to about 800 mL distilled water. After dissolution of ingredients, the final volume was completed to 1 L with distilled water. Final pH was adjusted to to 8.0, and the medium was sterilized by autoclaving at 121°C for 20 min. SS 6 and 7 were filter-sterilized and added aseptically to the sterilized medium.

B. Standard curve for total phenolic content assay
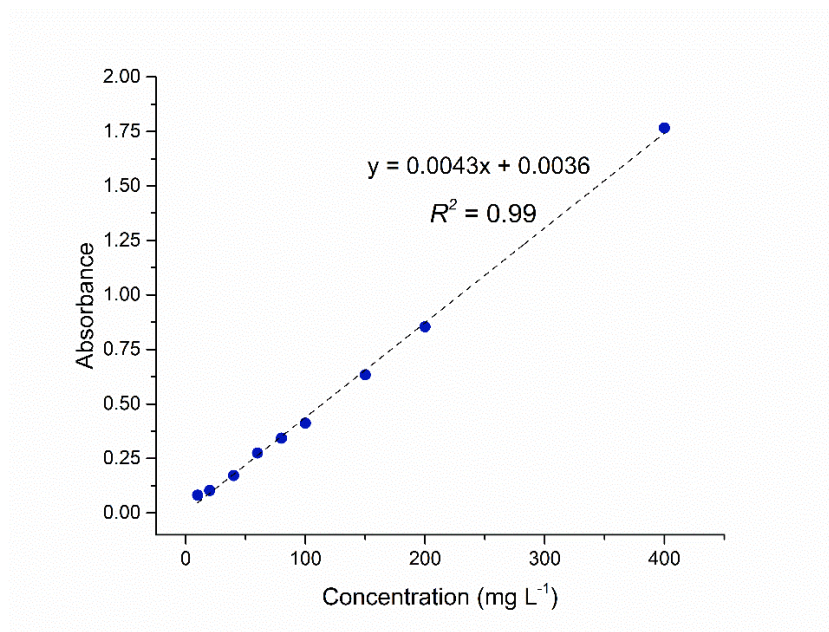

$y = 0.0043x + 0.0036$

$R^2 = 0.99$

Figure 6.1 The curve prepared with serially diluted gallic acid solutions. The regression equation used in calculation of gallic acid equivalents is shown on the regression line.

C. Standard curve for total flavonoid content assay

The figure shows a scatter plot with Absorbance on the y-axis (0.0 to 2.5) and Concentration (mg L^{-1}) on the x-axis (0 to 120), with a dashed regression line.
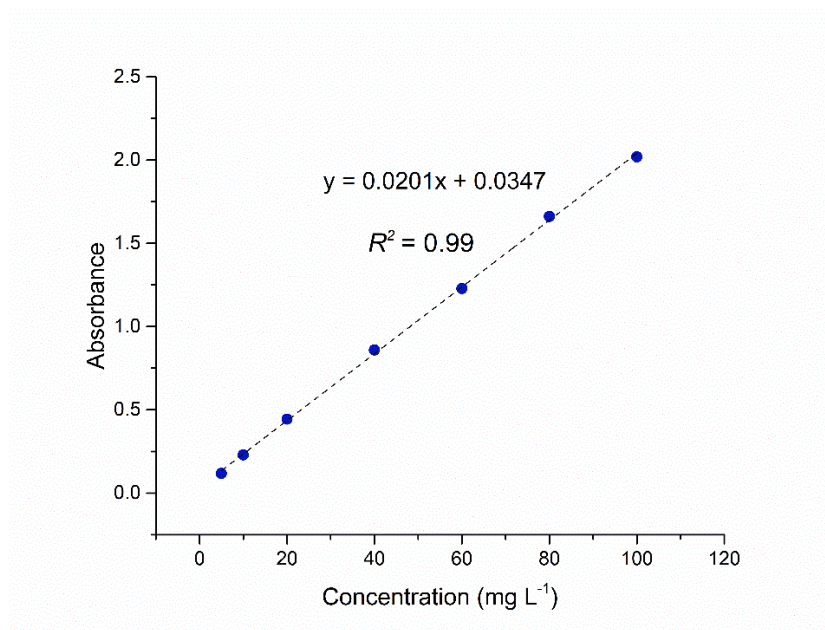
y = 0.0201x + 0.0347

R^2 = 0.99

Figure 6.2 The standard curve prepared with serially diluted quercetin solutions. The regression equation used in calculation of quercetin equivalents is represented on the regression line.
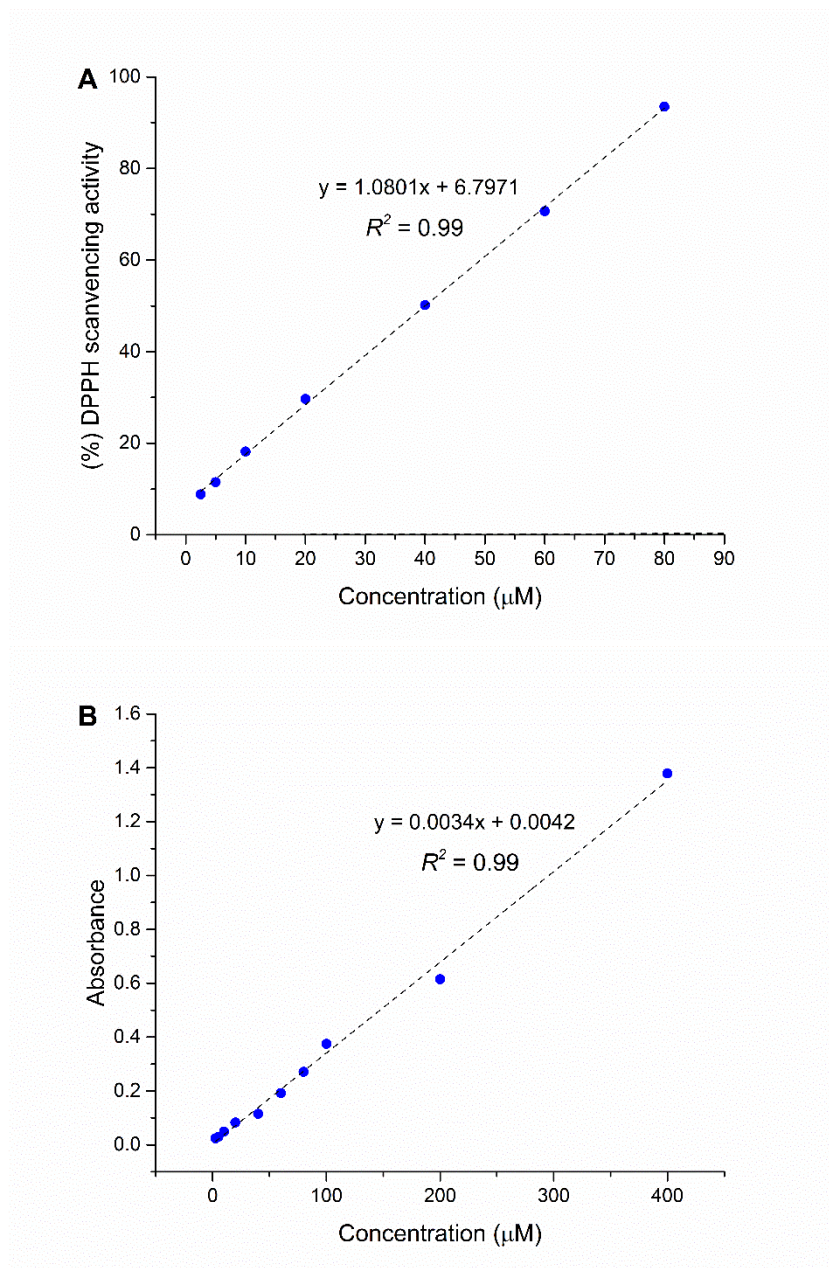
D. Standard curves for antioxidant assays

Figure 6.3 The standard curves of (A) DPPH assay and (B) FRAP assay prepared with serially diluted trolox solutions. The regression equations used in calculation of trolox equivalents are represented on the regression line.

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Onur, Bulut
Nationality: Turkish (TC)
Date and Place of Birth: 28 June 1989, Ankara
Marital Status: Single
Phone: +90 312 210 51 56
email: onnbulut@gmail.com

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biology	2014
BS	İstanbul University Molecular Biology and Genetics	2012
High School	80. Yıl High School, Aydın	2007

WORK EXPERIENCE

Year	Place	Enrollment
2015-Present	Konya Food and Agriculture University Dept. of Molecular Biology and Genetics	Research Assistant

FOREIGN LANGUAGES

English

PUBLICATIONS

1. **Bulut, O.**, Oktem, H. A., & Yilmaz, M. D. (2020). A highly substituted and fluorescent aromatic-fused imidazole derivative that shows enhanced antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Journal of Hazardous Materials, 122902.

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