T.C.

ÇANAKKALE ONSEKİZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ELUCIDATION OF THE EFFECTS OF FERMENTED *SPIRULINA* PROTEIN HYDROLYSATE AND BIOACTIVE PEPTIDE FRACTIONS ON ANTI-INFLAMMATORY, ANTICANCER, AND INDIRECT ANTIOXIDANT MECHANISMS FOR THERAPEUTIC AND NUTRACEUTICAL APPLICATIONS

MASTER OF SCIENCE THESIS

ZEYNEP ÖZLEM CİNAR

Thesis Supervisor
PROF. DR. TUĞBA TÜMER

Second Thesis Supervisor
PROF. DR. MEHMET AY

ÇANAKKALE – 2023

T.C.

ÇANAKKALE ONSEKİZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ELUCIDATION OF THE EFFECTS OF FERMENTED *SPIRULINA* PROTEIN HYDROLYSATE AND BIOACTIVE PEPTIDE FRACTIONS ON ANTI-INFLAMMATORY, ANTICANCER, AND INDIRECT ANTIOXIDANT MECHANISMS FOR THERAPEUTIC AND NUTRACEUTICAL APPLICATIONS

MASTER OF SCIENCE THESIS

ZEYNEP ÖZLEM CİNAR

Thesis Supervisor

PROF. DR. TUĞBA TÜMER

Second Thesis Supervisor

PROF. DR. MEHMET AY

ÇANAKKALE – 2023

T.C.
ÇANAKKALE ONSEKİZ MART ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

The study entitled **"Elucidation of The Effects of Fermented *Spirulina* Protein Hydrolysate and Bioactive Peptide Fractions on Anti-Inflammatory, Anticancer, and Indirect Antioxidant Mechanisms for Therapeutic and Nutraceutical Applications"** submitted by **Zeynep Özlem CİNAR** under supervision of Prof. Dr. Tuğba TÜMER was defended on **07/03/2023**. This thesis has been approved as **Master of Science** in **Molecular Biology and Genetics** of Çanakkale Onsekiz Mart University School of Graduate Studies by the Examining Committee Members.

Examining Committee

Prof. Dr. Tuğba TÜMER   …………………………
(Supervisor)
Assoc. Prof. Dr. Tuğba GÜNGÖR   …………………………
(Member)
Assoc. Prof. Dr. Müge İŞLETEN HOŞOĞLU   …………………………
(Member)   .

Tez No   : 10532306
Tez Savunma Tarihi : 07/03/2023

Assoc. Prof. Dr. Yener PAZARCIK

Director
School of Graduate Studies
/03/2023

PLAGIARISM DECLARATION PAGE

I declare that all the information and results offered in visual, and written form are obtained by myself observing the academic and ethical rules. Moreover, all other results and information referred to in the thesis but not specific to this study are cited.

Zeynep Özlem CİNAR
07/03/2023

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my supervisor, Prof. Dr. Tuğba TÜMER, for her patience, support, and inspiration as she shared her expertise with me during my studies. She included and trusted me in many scientific projects, her trust made me more confident and her inspiration made me want to be more productive throughout all my studies. Her leadership enhanced my scientific abilities and most importantly my ethical knowledge.

I want to sincerely thank Prof. Dr. Mehmet AY, my second supervisor, for educating me about the chemistry part of my thesis, for sharing his expertise, and for his encouragement.

I would like to thank Assoc. Prof. Dr. Tuğba GÜNGÖR for supporting me, sharing her knowledge with me, and for our little chats that gave me guidance during my chemistry laboratory work. Further, I would like to thank Assoc. Prof. Dr. Müge İŞLETEN HOŞOĞLU for giving me the opportunity to work on this project, I have learned and improved myself with this opportunity.

I would like to express that I am so grateful for working and sharing with our lab team, Adem, Berkay, Hazal, Serhat, and for my friends Buse, Hüma, Kübra, Raşit, Baran, Melek, and Ece. They have been my family in Çanakkale, we have shared all the joy and tears together all through these years.

Finally, my sincere gratitude goes to my family, my mom Nilüfer CİNAR, my dad Metin CİNAR, and my sister İrem Nur CİNAR. I am so lucky to have a family that is full of support and unconditional love. Any achievement I have is a product of their love and trust.

ÖZET

FERMENTE *SPİRULİNA* PROTEİN HİDROLİZATI VE BİYOAKTİF PEPTİT FRAKSİYONLARININ ANTİİNFLAMATUAR, ANTİKANSER VE İNDİREKT ANTİOKSİDAN MEKANİZMALAR ÜZERİNE ETKİLERİNİN TERAPÖTİK VE NUTRASÖTİK UYGULAMALAR İÇİN AYDINLATILMASI

Zeynep Özlem CİNAR

Çanakkale Onsekiz Mart Üniversitesi

Lisansüstü Eğitim Enstitüsü

Moleküler Biyoloji ve Genetik Anabilim Dalı Yüksek Lisans Tezi

Danışman: Prof. Dr. Tuğba TÜMER

İkinci Danışman: Prof. Dr. Mehmet AY

07/03/2023, 51

Artan metabolik sendrom oranı, inflamatuvar ve enfeksiyon hastalıkları nedeniyle takviye edici ürünlere ve sağlıklı beslenmeye olan ilgi giderek artmaktadır. Bu bağlamda, *Spirulina platensis*, sağlığa yararlı özellikleri nedeniyle yenilikçi nutrasötiklerin üretimi için en çekici kaynaklardan biri haline gelmiştir. Bu çalışmada, üç farklı mikrobiyal fermentasyon ile (*Lactobacillus helveticus, Kluyveromyces marxianus* ve *Lactobacillus helveticus* ve *Kluyveromyces marxianus*'un birlikte fermentasyonu) erlen düzeyinde gerçekleştirilen fermente *Spirulina* (FS) (FS by KM, FS by LH ve FS by mix) ürünlerinin, *Lactobacillus helveticus ve Kluyveromyces marxianus*'un birlikte fermentasyonu ile farklı koşullarda gerçekleştirilen (havalandırma 0.5 vvm (M2); havalandırma 1 vvm (M4); Cascade üretim (M6) ve sabit pH'da gerçekleştirilen üretim (M8)) biyoreaktör ürünlerinin, M6'nın ultrafiltrasyon (UF) işlemlerinden geçirilmiş fraksiyonlarının (>2 kDa, 2-5 kDa, 5-10 kDa), seçilen fraksiyonun (5-10 kDa) peptit fraksiyonlarının (FR1, FR2, FR3 ve FR4) ve fermente edilmemiş (kontrol) *Spirulina* ürünlerinin potansiyel antikanser ve NO baskılayıcı etkileri araştırılmıştır.

M6 ve FS by mix ürünlerinin, sitotoksik etki göstermeksizin, makrofajların, LPS ile indüklenen NO salınımını etkili bir şekilde azalttığı görülmüştür. Antikanser aktiviteler ele

alındığında ise, M6, M6 5-10 kDa fraksiyonu, M6 (5-10 kDa) fraksiyonuna ait FR1 ve FR2 fraksiyonları, HT-29 hücrelerinin canlılığını seçici olarak inhibe ederek umut vadeden etkiler göstermiştir. Ayrıca, M6 ve M6 (5-10 kDa) fraksiyonu, antioksidan genlerin (Nrf-2, HO-1, NQO1, Gclc ve Srnx1) ifadelerini mRNA seviyesinde arttırmıştır ve ürünlerin Nrf-2'nin nükleer akümülasyonunu stimüle ettiği saptanmıştır. Bu çalışmanın bulguları değerlendirildiğinde, FS by mix, M6, M6 5-10 kDa fraksiyonu, FR1 ve FR2'nin potansiyel antikanser, antioksidan ve NO baskılayıcı etkilerinin umut vadettiği çıkarımlanabilir. Fermente *Spirulina* hidrolizatları, daha kapsamlı analizler ile değerlendirilerek nutrasötikler için yeni bir içerik maddesi olarak geliştirilebilir.

Anahtar Kelimeler: *Spirulina platensis*, biyoaktif peptit fraksiyonları, antikanser, antioksidan, Nrf-2, fermentasyon

ABSTRACT

ELUCIDATION OF THE EFFECTS OF FERMENTED *SPIRULINA* PROTEIN HYDROLYSATE AND BIOACTIVE PEPTIDE FRACTIONS ON ANTI-INFLAMMATORY, ANTICANCER, AND INDIRECT ANTIOXIDANT MECHANISMS FOR THERAPEUTIC AND NUTRACEUTICAL APPLICATIONS

Zeynep Özlem CİNAR

Çanakkale Onsekiz Mart University

School of Graduate Studies

Master of Science Thesis in Molecular Biology and Genetics

Supervisor: Prof. Dr. Tuğba TÜMER

Co-supervisor: Prof. Dr. Mehmet AY

07/03/2023, 51

Supplementary products and healthy diets are arousing interest with the increasing metabolic syndrome rate, and inflammatory and infectious diseases. In this manner, *Spirulina platensis* is one of the most attractive sources for the production of innovative nutraceuticals due to its health-beneficial properties. Enhancing the impact of fermentation on *Spirulina platensis'* health-beneficial effects was demonstrated via *in vitro* studies in the scope of this thesis. Fermented *Spirulina* (FS) products, FS by Lactobacillus helveticus (FS by LH), FS by Kluyveromyces marxianus (FS by KM), and FS by the combined culture of both microorganisms (FS by mix); FS by LH and KM under different conditions (aeration 0.5 vvm (M2); aeration 1 vvm (M4); Cascade production (M6) and constant pH production (M8)) bioreactor products, ultrafiltration fractions of M6 (>2 kDa, 2-5 kDa, 5-10 kDa), peptide fractions (FR1, FR2, FR3, FR4) of selected UF product (5-10 kDa) and unfermented *Spirulina* (control) products were investigated in terms of their potential anticancer, and NO suppressive effects.

M6 and mix-fermented products efficiently decreased the LPS-induced NO secretion of macrophages without showing cytotoxic effects. In terms of anticancer activity, M6, M6 5-10 kDa, FR1, and FR2 showed promising effects by selectively inhibiting the viability of

HT-29 cells. Moreover, M6 and M6 5-10 kDa upregulated the expressions of antioxidant genes (Nrf-2, HO-1, NQO1, Gclc, and Srnx1) on mRNA level and promoted the nuclear accumulation of Nrf-2. On the whole, the findings of this study present a positive aspect on potential anticancer, antioxidant, and NO suppressive effects of the mix, M6, M6 5-10 kDa, FR1, and FR2. Hence, fermented *Spirulina* hydrolysates could be developed as a novel ingredient for nutraceuticals by further more comprehensive analysis.

Keywords: *Spirulina platensis*, bioactive peptide fractions, anticancer, antioxidant, Nrf-2, fermentation

TABLE OF CONTENT

Page No.

CHAPTER 5

CONCLUSION

ABBREVIATIONS

BAP	Bioactive Peptide Fraction
IL-1β	Interleukin-1 Beta
TNF-α	Tumor Necrosis Factor Alpha
NO	Nitric Oxide
ROS	Reactive Oxygen Species
ARE	Antioxidant Responsive Element
NQO1	NADPH quinone oxidoreductase-1
HO-1	Heme oxygenase-1
Nrf-2	Nuclear factor erythroid 2-related factor 2
Gclc	Glutamate-Cysteine Ligase Catalytic Subunit
Srnx1	Sulfiredoxin-1
UF	Ultrafiltration
RP-HPLC	Reverse-Phase High-Performance Liquid Chromatography
KM	*Kluyveromyces marxianus*
LH	*Lactobacillus helveticus*
LPS	Lipopolysaccharide from *E. coli*
DOX	Doxorubicin
SUL	Sulforaphane
SI	Selectivity Index
IC$_{50}$	Half maximal inhibitory concentration
SRB	Sulforhodamine B
MTT	3-(4,5-dimethylthiazol-2-yl) 22,5-diphenyltetrazolium bromide
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
ACN	Acetonitrile
TFA	Trifluoroacetic Acid

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

The recognition of supplementary products and healthy diets is arousing with the increasing metabolic syndrome rate, and inflammatory and infectious diseases, especially after the pandemic. Proteins are one of the most important components in human and animal nutrition and have proven to have biologically active constituents that hold health-promoting effects beyond their properties as rich nutrients. Bioactive peptides (BAPs) are specific sequences of amino acids with several nutraceutical/therapeutic properties and potential applications that can exert their action via *in vitro* or *in vivo* hydrolysis (Korhonen & Pihlanto, 2006). Although the bioactive peptides may derive from a variety of protein sources, microalgae-derived proteins have been recognized due to their plenitude and amino acid composition (Z. Wang & Zhang, 2016b). As a protein source, microalgae species contain a similar amount of protein along with animal-derived sources. Yet, microalgae extractions are more advantageous in terms of nutritional content including vitamins, minerals, and carotenoids within the proteins (Koyande et al., 2019). *Spirulina platensis* is blue-green algae (cyanobacterium) living in water and it is known that *Spirulina* has been a popular ingredient for nutritional supplements due to its content of abundant proteins, carotenoids, essential fatty acids, vitamin B, vitamin E, and minerals. In addition, they have been identified for their significant health benefits such as being antimicrobial and antioxidant. Most of these beneficial activities have been attributed to the bioactive peptide content of *Spirulina* (Czerwonka et al., 2018; Dartsch, 2008; El-Baz et al., 2013; Koníčková et al., 2014). Since *Spirulina* is rich in bioactive peptides; its potential use in functional foods, cosmetics, and pharmaceuticals was investigated. Nonetheless, because of high manufacturing costs and a lack of bioactivity research, the manufacturing of bioactive peptides produced from *Spirulina* has not yet been accomplished (Lafarga et al., 2021a).

1.1. Inflammation, Inflammatory Diseases, and Cancer

Inflammation is a complicated protective response against toxins, infections, or injuries that leads to healing by starting a cascade of events in the tissue and is divided into two types according to its duration. While inflammation that lasts less than 48 h is considered acute inflammation, the other type lasting more than 48 h is accepted as chronic inflammation. (Ansar & Ghosh, 2016). In the acute stage, inflammatory cells are recruited to the site of a wound, where they release pro-inflammatory mediators such as cytokines and chemokines. In this period, anti-inflammatory agents are also secreted by the immune system to prevent excessive inflammation that can lead to tissue damage and delayed healing. In the most severe cases of inflammation, certain immune cells are released, such as neutrophils, macrophages, and lymphocytes. These cells play key roles in the destruction of pathogens and the induction of an immune response. However, these highly reactive cells also cause significant damage to the surrounding tissue and can worsen the damage resulting from the initial injury. Therefore, it is important to regulate the activity of these cells to ensure their proper function and minimize undesirable side effects (Ansar & Ghosh, 2016).

Earlier studies provide shreds of evidence for the role of chronic inflammation in several human diseases such as cardiovascular diseases, metabolic syndrome, inflammatory bowel disease, central nervous system (CNS) diseases, and different types of cancer since prolonged inflammation can become pathologic (Panigrahy et al., 2021). In inflammation interleukin-1s (IL-1) are known as the main downstream mediator and there are two IL-1s; IL-1α, and IL-1β. In the synthesis of IL-1β, TNF-α, IL-18, IL-1α or IL-1β itself can play the role of stimuli (Dinarello & van der Meer, 2013). Unregulated production of IL-1β observed in inflammatory diseases and also proved to be effective in the progression of the disease. Besides inflammatory diseases, the overproduction of IL-1β is also involved in invasive cancer types and it is already known that chronic inflammation is recognized as one of the major causes of cancer, and tumorigenesis (Bent et al., 2018). Also, cytokines other than IL-1β; such as TNF-α, and IL-6 have a key effect on the progression of cancer by enhancing the invasiveness of malignant cells (Balkwill et al., 2005). In a clinical study conducted with 30 ovarian cancer patients to show the linkage between inflammation and ovarian cancer, the effects of TNF-α on tumor growth and invasion were investigated. In blood samples of patients after treatment with a TNF-α inhibitor, etanercept, it was reported that immunoreactive TNF-α levels were increased while the IL-6 levels were significantly

decreased. Allied with provided data, targeting inflammatory cytokine TNF-α in the treatment of ovarian cancer is pointed out as a possible strategy that needs to be confirmed with further studies (Madhusudan et al., 2005). In a recent clinical study, similar shreds of evidence were demonstrated for the role of the IL-1β pathway in lung cancer. This study indicated that targeting the IL-1β pathway may have importance in delaying disease progression (Wong et al., 2020). In the view of earlier studies, targeting inflammatory pathways is a conventional treatment strategy in the improvement of de novo therapeutics for the treatment and prevention of inflammatory diseases and cancer types. Several cytokines and molecules have key importance in quantifying inflammatory responses or determining the anticancer properties of test samples in ongoing studies. Nitric oxide (NO) is involved in several physiological and pathophysiological pathways by using multiple classes of nitric oxide synthase (NOS) enzymes, therefore, it carries out a substantial role in the life of many organisms (Sun et al., 2003). NO is one of the main regulator molecules in inflammation that can be generated by mammalian cells. Excessive production of NO may also cause toxicity in the cell instead of regulating the host defense mechanisms (Tripathi et al., 2007). Hereupon, quantification of NO released from an LPS-induced macrophage model to evaluate the inflammatory response is one of the commonly used approaches and Griess assay is a well-established method for this purpose (Dirsch et al., 1998).

1.2. Antioxidant and Detoxification Genes

Reactive oxygen species (ROS), which are formed by cells as byproducts of their metabolic activities during the oxidative reaction process of the mitochondrial respiratory chain, have a significant influence on the physiology of cells. Regulated levels of ROS, which are primarily produced by mitochondria and the cytochrome P450 (CYPs) enzyme system, have advantageous effects like the destruction of pathogens and the healing of wounds. As the oxygen and metabolic levels rise, more ROS are produced, and as the permeability of the mitochondrial membrane increases, more ROS are released into the cytosol. The metabolic process of xenobiotics catalyzed by CYPs can be another significant source of ROS. A regulated ROS level is essential for maintaining the body's homeostasis since ROS are significant signaling molecules engaged in a number of processes. Overexposure to ROS disrupts redox equilibrium, leading to oxidative stress and ROS-

mediated harm to vital organelles and macromolecules including DNA and proteins, as well as harm associated with aging, cancer, diabetes, and neurodegeneration (He et al., 2017; Yang & Lian, 2020). In this case, the antioxidant defense system becomes involved in balancing oxidative stress caused by ROS overproduction by the upregulation of antioxidant detoxification genes and protecting the cell from oxidative stress. Moreover, a set of antioxidant detoxification genes regulated by antioxidant responsive element (ARE) including glutathione S-transferase (GST), NADPH quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), and ferritin H (FH) upregulated on the transcriptional level and one of the main transcription factor for ARE activation is NFE2-like 2 (Nrf-2) (Ray et al., 2012). Nrf-2 act as a mediator for detoxification genes NADPH quinone oxidoreductase-1 (NQO1), Glutamate-cysteine ligase catalytic subunit (Gclc), and antioxidant genes heme oxygenase-1 (HO-1), sulfiredoxin-1 (Srxn1). Detoxification genes clear the redox environment by detoxifying ROS and inducing the production of antioxidant forms of molecules and glutathione. Also, the antioxidant gene HO-1 reduces the production of ROS and stimulates mitochondrial biogenesis (Chen & Maltagliati, 2018; Otterbein et al., 2016). In normal conditions, Nrf-2 situates in the cytoplasm of the cell; under oxidative stress or with a presence of an activator it translocates into the nucleus.

1.3. Novel and Sustainable Nutrition

The notion of food is changing in modern civilizations, and people are demanding dietary products with additional health benefits that go beyond delivering essential nutrients. Since poor eating habits are one of the numerous risk factors for chronic diseases (CD), there is substantial evidence to support the significance of nutrition in human health (Domínguez Díaz et al., 2020). For this reason, producers focused on innovative dietary supplements, though the growth of the development of new functional food items is becoming more difficult because of the consumer's need for nutritious products with health benefits (Shah, 2007). There are several technologies including traditional and innovative methods that have evolved substantially to keep physiologically active substances from degrading. Along with the knowledge on the significance of the link between structure and property; the activity of dietary products depends on active components' integrity and accessibility to the target site after consumption. Yet, the bioactivity of dietary products is complicated since foods are

mostly intricate mixes of macro and micro components that may either trap an active molecule or modify its release or inhibit its activity. On the other hand, global hunger risk is increasing with the growth in population and these circumstances show that novel and sustainable nutrient sources are in need (Betoret et al., 2011).

1.3.1. *Spirulina Platensis*

Microalgae are plant-like organisms with diameters between 1 and 50 micrometers also known as phytoplanktons. Recently, it is known that there are 200,000 to 800,000 different species of microalgae, and not all the species are described or classified (Wolkers et al., 2011). A significant number of microalgae species have been shown to provide comparable levels of protein to commonly consumed protein sources such as milk, soy, eggs, and meat; nevertheless, protein extraction from microalgae may be considered more advantageous in terms of variable nutritional content, efficiency, and yieldance (Wallace, 2000). Microalgae range in extreme environmental circumstances that cause oxidative stress, and due to these conditions and their phototrophic nature, microalgae generated natural defense mechanisms such as the synthesis of antioxidants and pigments (for example chlorophylls, carotenes, and phycobiliproteins). Since humans cannot generate these substances on their own, they are helpful as supplements (Koyande et al., 2019). Moreover, microalgae contain bioactive compounds known as high-value molecules like bioactive peptides (BAPs), polyunsaturated fatty acids (PUFAs), vitamins, polyphenols, and phytosterols making it a valuable nutraceutical source. Consequently, *Spirulina* is composed of nearly 60% protein with 22 essential amino acids. These proteins have significant biological activities and can be hydrolyzed into bioactive peptides. Bioactive peptides, which may be derived from a variety of protein sources, are particular sequences of amino acids with biological activity that have a variety of health benefits and potential uses. Several microalgae species including *Spirulina plantesis* have been used in the industry of healthy food products and supplementary products commonly (Koyande et al., 2019). Since *Spirulina* is rich in bioactive peptides; its potential use in functional foods, cosmetics, and pharmaceuticals was investigated. However, because of high manufacturing costs and a lack of bioactivity research, the industrial production of bioactive peptides produced from *Spirulina* has not yet been accomplished (Lafarga et al., 2021b).

Spirulina plantesis gained more popularity in recent years as the studies showing its pharmaceutical potential by elucidating the antioxidant, immunomodulatory, anti-inflammatory, anticancer, antiviral, and antibacterial activities increased. The possible antioxidant action of *Spirulina* is also investigated and seen that it stimulates the activity of antioxidant enzymes and scavenges free radicals in addition to its protective effects against DNA damage and lipid peroxidation. Significant antioxidant effects of *Spirulina* were also shown in a number of clinical studies. Besides, the regulatory effects of *Spirulina* on cytokines involved in inflammatory pathways such as IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α indicated (Q. Wu et al., 2016). In a case study signifying the potential use of *Spirulina* in cancer treatment conducted by Kızıltan et al., (2015), a female patient with a vulvar tumor received a combined therapy of *Spirulina plantesis* and metronidazole along with radiotherapy. Tumor regression as a response to radiotherapy was observed with no adverse effects (Kiziltan et al., 2015). At the moment, many studies are focusing to achieve improvement in health beneficial effects and bioavailability of *Spirulina plantesis*.

1.3.2. Fermentation

Bioactive peptide concentrates with certain biological activities have become important components for supplementary products and/or pharmaceutical preparations and are often generated by proteolysis which is the hydrolysis process of proteins via particular enzymes. Although the bioactive peptides are released during digestion after consumption of foods rich in protein, the type and amount of peptides cannot be controlled and may be insufficient to produce important therapeutic effects. In this manner, bioactive peptide concentrates have been obtained synthetically or by using enzymatic and recombinant methods. For enzymatic hydrolysis, it is crucial to select the convenient proteolytic enzyme and arrange the ideal physicochemical condition (Samarakoon & Jeon, 2012); (Lee et al., 2010). On the other hand, fermentation is an alternative way to generate bioactive peptides and it is a natural, low-cost, and sustainable process compared to the generally used methods such as enzymatic hydrolysis so it can be an advantageous alternative method for this purpose. Fermentation is a process that has been used to increase the nutritional value of

foods, preserve the flavor profile or make them more attractive for years. It is known that as a result of fermentation bioactive peptides are formed by the hydrolysis of some proteins in foods (Dallagnol et al., 2012); (Jakubczyk et al., 2013); (Jhan et al., 2015); (Moayedi et al., 2016). As a result of fermentation, indigestible components of the original product can be hydrolyzed into smaller molecules and enhance the bioavailability and digestibility of the food. Correspondingly, the process varies the taste profile of food and improves health-beneficial effects including anti-inflammatory activity besides reducing harmful and toxic molecules (Garofalo et al., 2022). As an example, kefir is a popular drink produced by fermentation with many health-improving effects including anticarcinogenic effects, reducing effects on inflammatory bowel diseases, and curative effects on neurodegenerative diseases. The therapeutic effects of kefir have been attributed to its antioxidant capacity and the bioactive compounds it contains and these benefactory molecules are produced by the fermentation process (Vieira et al., 2021).

4.1. Scope of Thesis

The general aim of this study is to investigate the potential anti-inflammatory, anticancer, and indirect antioxidant effects of fermented *Spirulina* protein hydrolysate and bioactive peptide fractions as well as to elucidate the mechanism of actions at the molecular level. *Spirulina* hydrolysates are produced by fermentation and fractionated through ultrafiltration (UF) by Assoc. Prof. Dr. Müge İşleten Hoşoğlu and her team due to collaboration with the TUBITAK project (218M289). Further fractionation of UF samples was accomplished in the scope of this thesis under the supervision of Prof. Dr. Mehmet Ay, also a part of the aforementioned project. Bioactivity and mechanisms underlying are not elucidated in the literature since the hydrolysates are obtained by novel optimized fermentation conditions; in the process of optimizing the fermentation conditions, bioactivity guidance was followed. This study includes the examination of fermented/unfermented products in terms of cytotoxicity and potential anticancer, anti-inflammatory, indirect

antioxidant properties, separation, and determination of the hydrolysate fraction with the most promising bioactivities.

Figure 1. Fermented *Spirulina* products investigated in this study.
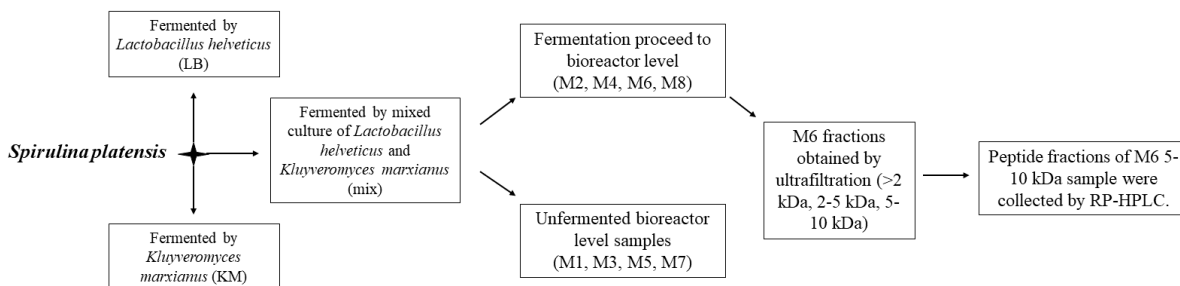
In this manner, the steps of this study are listed below.

- ✓ Screening of cytotoxicity and NO suppressive effects of three fermented *Spirulina* (FS) products (FS by *Kluyveromyces marxianus*, by FS *Lactobacillus helveticus*, FS in the combination of both *Kluyveromyces marxianus* and *Lactobacillus helveticus*) and determination of the most promising hydrolysate.

- ✓ Screening of cytotoxicity and NO suppressive effects of different bioreactor products of FS in the combination of both *Kluyveromyces marxianus* and *Lactobacillus helveticus* (M2 (aeration 0.5 vvm), M4 (aeration 1 vvm), M6 (Cascade production), M8 (constant pH production)).

- ✓ Elucidation of potential anticancer effects of chosen bioreactor product (M6) by IC_{50} screening.

- ✓ Screening of cytotoxicity and NO suppressive effects of three M6 fractions obtained by UF (>2 kDa, 2-5 kDa, 5-10 kDa) and determination of UF fraction with promising bioactivities.

- ✓ Separation of 5-10 kDa UF fraction to peptide fractions by RP-HPLC.

- ✓ Screening of cytotoxicity and NO suppressive effects of four RP-HPLC fractions (FR1, FR2, FR3, FR4).

- ✓ Elucidation of anti-inflammatory anticancer, and indirect antioxidant effects of M6, 5-10 kDa UF fraction, and FR1 via qPCR by using specific probes.

- ✓ Quantification of Nrf-2 protein expression to elucidate indirect antioxidant effects of M6 and 5-10 kDa UF fraction via Western Blot.

8

These findings are novel since there is no evidence related to the biological activities of these products in the literature.

CHAPTER 2
PREVIOUS STUDIES

There was no data in the literature relevant to the products studied in this thesis since all hydrolysates were obtained by novel optimized fermentation process conditions as part of the TUBİTAK 218M289 project. Though, there are several studies that investigated the bioactivity of *Spirulina platensis* hydrolysates obtained via enzymatic hydrolysis or fermentation aside from the studies on components of *Spirulina platensis*.

Anticancer properties of *Spirulina platensis* water extract were examined on the Caco-2 (human colorectal adenocarcinoma) cell line and the study indicated that *Spirulina platensis* upregulated the expression of the pro-apoptotic gene Bax while downregulating the expression of anti-apoptotic gene Bcl-2. In this study, the inhibitory effects of *Spirulina* on cell migration and growth were also demonstrated (Smieszek et al., 2017).

In the study of Vaňková et al., (2018), the effects of chlorophylls from *Spirulina platensis* on human pancreatic adenocarcinoma cell lines (PaTu-8902, MiaPaCa-2, BxPC-3) were examined. Compounds showed a more specific inhibitory effect against PaTu-8902 cells. These potential antiproliferative and anticancer effects were attributed to the antioxidant capacity of *Spirulina platensis* (Vaňková et al., 2018).

In an *in vitro* study, purified polysaccharide SPS-3-1 from *Spirulina platensis* was investigated in terms of anticancer and health-promoting effects. The protective effects of SPS-3-1 on the intestinal epithelial barrier were indicated by damaging Caco-2 cells via H_2O_2 with and without the presence of SPS-3-1 and in polysaccharide presence decomposition of the cell monolayer was reduced. Moreover, SPS-3-1 was proven to have antiproliferative effects on Caco-2 and HepG2 (human hepatoma cell line) cancer cell lines resulting that *Spirulina* may be used in the production of nutraceuticals (Q. Wang et al., 2020).

Wang and Zhang, (2016), proteins were extracted from *Spirulina platensis* and hydrolyzed via proteolytic enzymes and >3, 3-5, 5-10, and <10 kDa fractions were collected

via UF. Cytotoxic effects of both hydrolysates and UF fractions were screened by 3-(4,5-dimethylthiazol-2-yl) 22,5-diphenyltetrazolium bromide (MTT) assay on human hepatoma cells (HepG-2), breast cancer cells (MCF-7), gastric cancer cells (SGC-7901), lung cancer cells (A549), colon cancer cells (HT-29) and immortalized liver cells (L-o2). Two of the polypeptides obtained by the trypsin hydrolysis showed a distinguished and selective inhibitory effect on cell growth of HT-29 cells with the IC_{50} value of ≤31.25 µg/mL; indicating that *Spirulina platensis* hydrolysates have a promising potential as an ingredient for nutraceuticals and/or as a therapeutic in pharmaceutics (Z. Wang & Zhang, 2016b).

In the following study performed by Wang and Zhang (2016), proteins were extracted from *Spirulina platensis* and were hydrolyzed by using a sequential method including two enzymes (alcalase and papain) and hydrolysates separated into >3, 3-5, 5-10, and <10 kDa fractions were collected via UF. The inhibitory impact of enzymatic hydrolysate was estimated on the following cancer cell lines: HepG-2, MCF-7, SGC-7901, A549, and HT-29. Hydrolysate showed a significant inhibitory effect on the viability of MCF-7, A549, and HT-29 cells at 500 µg/mL dose. Moreover, >3, and <10 kDa fractions in all UF fractions were determined as the ones with the highest potency in terms of anticancer activity. >3 kDa UF fraction was further purified into smaller fractions through gel chromatography and fractions were also investigated to elucidate their cytotoxicities on cancer cells and determine the active fractions. Active peptide fractions were characterized and anti-tumor activities were estimated *in vivo* and *in vitro*. Results indicated that *Spirulina* hydrolysate has the potential to be developed as a therapeutic agent for tumor inhibition and/or as a bioactive food ingredient (Z. Wang & Zhang, 2016a). Also, a similar study conducted with *Spirulina* hydrolysate was obtained by using three-stepped enzymatic hydrolysate (pepsin, trypsin, and chymotrypsin). The bioactive peptide was determined from whole hydrolysate and characterized. The specific sequence of the peptide was attributed to have selective significant inhibitory activity on the HT-29 cancer cell line with the IC_{50} value of 99.88 µg/mL suggesting may be considered in further studies for development (Z. Wang & Zhang, 2017).

Recently, Jamnik et al., 2022, investigated the enhancing effects of fermentation on the bioactivity of *Spirulina platensis*. Lactic acid fermentation was accomplished with *Lactobacillus plantarum*. Even though there was no change in protein composition after

fermentation, a raise in non-protein nitrogen amount was observed pointing out that fermented product contains proteins with higher bioavailability. In terms of bioactivity analysis, fermentation enhanced the direct antioxidant activity of *Spirulina* and its polyphenol content was implied as responsible for the antioxidant capacity. Furthermore, preventive effects of the fermented product on lipid peroxidation of *Saccharomyces cerevisiae* were shown. Results were correlated with the potential use of fermented *Spirulina platensis* for nutraceuticals (Jamnik et al., 2022).

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals, Kits, and Essential Items

Chemicals, kits, and essential items used through experimental procedures were listed below in Table 1.

Table 1

List of chemicals, kits, and essential items.

Cell Culture Studies	Hydrochloric acid (320331)	Protein and RNA Quantification Studies
Dulbecco's Modified Eagle's Medium-high glucose (D6429)	Sodium pyruvate (P5280)	TaqMan™ Fast Advanced Master Mix (4444557)
Dulbecco's PBS (PBP01)	Trisma base (T1503)	TaqMan™ Probes: β-actin, caspase-3, Bax, Bcl-2, HO-1, NQO1, Nrf-2, Gclc, Srnx1
RPMI-1640 Medium (R6504)	Ultra Pure™ Dnase/Rnase-free Distilled water (10377035)	Western Blotting Filter Paper, Extra Thick (88620)
Dulbecco's Modified Eagle's Medium without phenol red (DMP17)	Gibco™ Amphotericin B (15290018)	Nitrocellulose Membrane, 0.45μm (88018)
Fetal Bovine Serum (10270106)	MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) (M5655)	SuperSignal™ West Pico PLUS Chemiluminescent (34580)
Penicilin-Streptomycin (15140122)	Sulforhodamine B sodium salt (S1402)	10X Bolt™ Sample Reducing Agent (B0009)
Glutamax™ Supplement (35050061)	**Kits**	Pierce™ LDS Sample Buffer, Non-Reducing (4X) (84788)
Trypsin-EDTA (0.25%), phenol red (25200056)	High-capacity cDNA Reverse Transcription Kit (4368814)	Prestained Protein Ladder (26619)
Doxorubicin hydrochloride (D2975000)	Pierce™ BCA Protein Assay Kit (23227)	Blotto, Non-Fat Dry Milk (sc-2324)
Sulforaphane (10496)	Total RNA Purification Plus Kit (48300)	Antibodies: Nrf-2 (sc-365949), β-actin (sc-47778)
Dimethyl sulfoxide (D2650)	QUBIT RNA BR Assay Kit (Q10210)	m-IgGκ BP-HRP (sc-516102-CM)
Lipopolysaccharides from E.coli O55:B5 (L6529)	IL-1β Mouse ELISA Kit (BMS6002)	**RP-HPLC Studies**
Acetic acid (1.00056.2500)	TNF-α Mouse ELISA Kit (BMS607-3)	Acetonitrile, >99.9% (34851)
Tryphan blue solution (T8154)	NE-PER™ Nuclear and Cytoplasmic Extraction Kit (78835)	Trifluoroacetic acid, >99.0% (302031)

3.1.2. Equipment

Equipment that was used for experiments and analysis (Table 2) in the scope of this thesis is presented in the Molecular Biology and Genetics Research Laboratory and Natural Products and Drug Research Laboratory at the Çanakkale Onsekiz Mart University (COMU).

Table 2

Equipment list.

Brand	Model	Equipment
Esco	CCL-170T-8	Cell Culture CO_2 Incubator
Esco	Class II BSC	Biological Safety Cabinets
BioRad	T100™	Thermal Cycler
Thermo Fisher Scientific	Applied Biosystems™ 7500	Real-Time PCR Systems
Thermo Fisher Scientific	Invitrogen Mini Blot Module	Wet Transfer Device
Thermo Fisher Scientific	Invitrogen Mini Gel Tank	Mini Gel Electrophoresis System
BioRad	-	Vertical Electrophoresis System
Hettich	MIKRO 200R	Refrigerated Micro Centrifuge
Hettich	Universal 320R	Standart Centrifuge
BioSan	TS-100	Thermo-Shaker
TECAN	Infinite 200 PRO	Microplate Reader
Scientific Industries	Genie 2	Vortex
Invitrogen	Qubit 4	Fluorometer
Major-Science (MS)	MW-23	Waver Shaker
Teknokroma	Europa Peptide C18	HPLC Peptide Column
Teknokroma	Peptide Guard Kit C18	Peptide Guard Column
Shimadzu	Prominence LC-20AR	Semi-preparative HPLC System
BUCHI	Interface I-300	Rotary Evaporator System

3.1.3. Cell Lines and *Spirulina platensis* Samples

All the cell lines studied for this thesis are listed in Table 3 and growth conditions are explained in method parts.

Table 3

List of cell lines.

Cell Line	Species	Type-Disease Model
HT-29	Homo sapiens	Colorectal adenocarcinoma
MCF7	Homo sapiens	Breast adenocarcinoma
PC-3	Homo sapiens	Prostatic adenocarcinoma
HUVEC	Homo sapiens	Normal
RAW264.7	Mus musculus	Macrophage

Fermented and unfermented *Spirulina* (FS and unFS) samples, ultrafiltration, and RP-HPLC fractions were listed below in Table 4.

Table 4

Classification of *Spirulina platensis* samples.

Samples	Types
LH, KM, and mix	Optimization Fermentation Products
C-LH, C-KM, and C-mix	Unfermented Control Products
M2, M4, M6, and M8	Bioreactor Fermentation Products
M1, M3, M5, and M7	Unfermented Control Products
M6: >2 kDa, 2-5kDa, and 5-10 kDa	Ultrafiltration Fractions of M6
M5: >2 kDa, 2-5kDa, and 5-10 kDa	Ultrafiltration Fractions of M5
FR1, FR2, FR3, FR4	RP-HPLC Fractions of M6 5-10 kDa

3.2. Methods

In the scope of this thesis, fermented *Spirulina* (FS) products by *L. helveticus* B-4526, by *K. marxianus* Y-329, and a combination of both *L. helveticus* and *K. marxianus* where *Spirulina* biomass used as a sole source of nutrient in a medium were investigated in terms of their effects on the viability of colon cancer cells and the NO release of LPS-induced macrophages. Then, with the chosen microorganisms bioreactor productions were performed under 4 different 3-L bioreactor conditions by our collaborative research group, and samples of 4 different bioreactor level FS products (M2, M4, M6, and M8) and their unFS counterparts (M1, M3, M5, and M7) were sent to our research group. Effects of bioreactor

FS products and their unfermented correspondings on the viability of HT-29 and HUVEC cells were evaluated. Then the bioreactor sample with the promising potential bioactivities was chosen to continue for fractionation steps (RP-HPLC) with the aim of determining active peptide fractions. Before the fractionation steps (RP-HPLC), Ultrafiltration (UF) was applied to the chosen bioreactor product and bioactivity studies continued with its three different UF fractions (>2 kDa, 2-5 kDa, 5-10 kDa). Then, as the last step of this bioactivity-oriented fractionation process, the UF fraction with the promising bioactivity was determined and this UF fraction was used for further fractionations done by RP-HPLC. Toxic/nontoxic doses of peptide fractions collected by RP-HPLC on cancer cell models were determined by SRB assay and mechanism studies completed by qPCR. Additionally, the potential antioxidant activities of samples were investigated via qPCR and Western Blot studies.
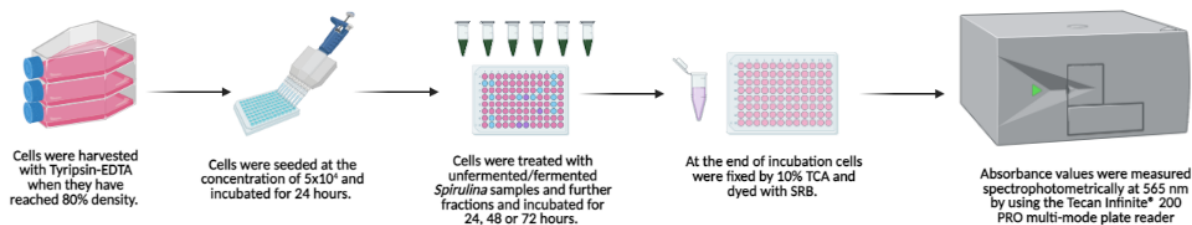
3.2.1. Cell Lines and Culture Conditions

HT-29 (human colorectal adenocarcinoma cell line), MCF7 (human breast cancer cell line), PC-3 (human prostate cancer cell line), and HUVEC (human umbilical vein endothelial cells) in DMEM containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin, 100 μg/mL streptomycin and incubated at 37°C under a saturating humidity atmosphere of 5% CO_2/95% air. In the same incubation conditions, RAW264.7 (mouse macrophage cell line) was cultured in RPMI supplemented with 10% (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

3.2.2. SRB Assay and IC$_{50}$ Determination

The toxic and non-toxic doses of unfermented/fermented *Spirulina* samples and further fractions were detected by SRB assay in each cell line. Cells that were seeded with the concentration of 5×10^4 were incubated for 24, 48, and 72 h with different doses of unfermented/fermented *Spirulina* samples and further peptide fractions. After the incubation periods, the old media of each well was removed and cells were fixed by using 100 μL of 10% (w/v) trichloroacetic acid (TCA) solution for each well for 1 hour at +4 °C. After the fixation procedure, wells were washed with distilled water 5 times and will be dried at room temperature (RT). 100 μL of 0.04% (w/v) SRB dye solution was added to each well and incubated at room temperature for 30 minutes and then the plate was washed with 1% (v/v) acetic acid solution 4 times and dried using a drier. 200 μL of 10 mM Tris Base solution was

added into each well to dissolve the SRB dye which connected the proteins and plate left on a constant speed shaker for 10 minutes at RT. After this process, the absorbance values were

Cells were harvested with Tyripsin-EDTA when they have reached 80% density.

Cells were seeded at the concentration of $5x10^4$ and incubated for 24 hours.

Cells were treated with unfermented/fermented *Spirulina* samples and further fractions and incubated for 24, 48 or 72 hours.

At the end of incubation cells were fixed by 10% TCA and dyed with SRB.

Absorbance values were measured spectrophotometrically at 565 nm by using the Tecan Infinite® 200 PRO multi-mode plate reader

measured spectrophotometrically at 565 nm by using the Tecan Infinite® 200 PRO multi-mode plate reader. IC_{50} values of the compounds were determined by GraphPad Prism 8 software.

Figure 2. Experimental steps of SRB Assay.

3.2.3. Griess Assay

Griess assay was conducted to investigate the effects of unfermented/fermented *Spirulina* samples and further fractions on nitric oxide (NO) release from LPS-induced mouse macrophages. RAW 264.7 were seeded at the concentration of $5x10^4$ in a 24-well plate and incubated for 24 h. Then samples and LPS (1 µg/mL) were treated and further incubated for 24 h. At the end of the incubation, the media of cells was collected. Sodium nitrite ($NaNO_2$) solutions were prepared in the range of 0-100 µM concentration to generate a standard curve. Then, supernatants and standards were placed into wells of a 96-well plate, mixed with Griess reagent (1:1), and incubated for 10 minutes at RT and in a dark environment. The amount of nitrite present in the medium was determined via the measurement of the formed color at 520 nm using a spectrophotometer with a microplate reader and the viability of cells was determined by MTT assay for the normalization.

3.2.4. MTT Assay

After collection of cell medium for Griess assay, 980 µL fresh media (no-phenol red DMEM) was added above cells and 20 µL MTT solution (0,2 mM/mL) was treated to each well and incubated for 2 h. Formazan crystals formed in wells dissolved within dimethyl sulfoxide (DMSO). Absorbances were quantified spectrophotometrically at 520 nm with the

reference wavelength of 620 nm by using the Tecan Infinite® 200 PRO multi-mode plate reader.
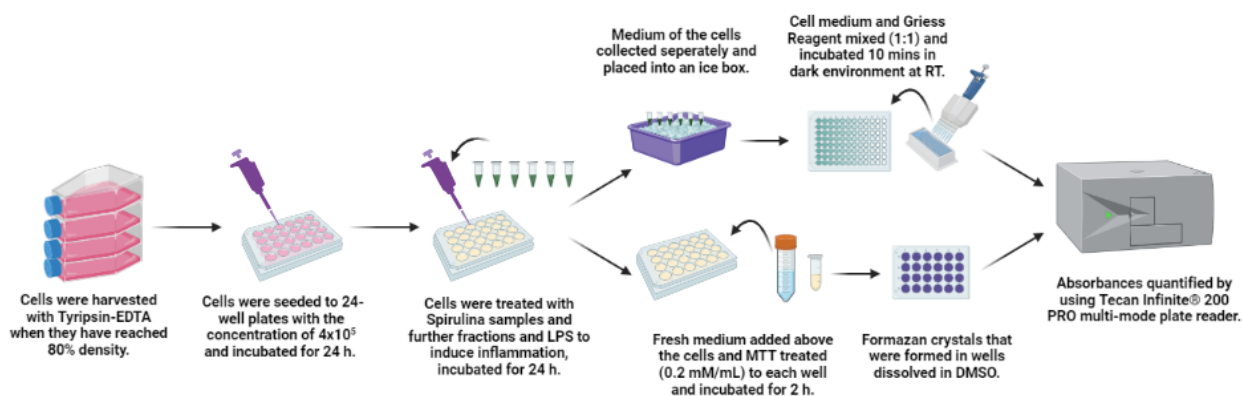

Figure 3. Experimental steps of Griess and MTT Assay.

3.2.5. Gene Expression Analysis

For the assessment of antioxidant, and anticancer effects of products on transcription level qPCR analysis were performed. After the treatment with *Spirulina* products, RNA samples were isolated from HT-29 and RAW264.7 cells by using NORGEN Total RNA Purification Plus Kit, and cDNA synthesis was carried out by using High-capacity cDNA Reverse Transcription Kit. Effects of the fermented *Spirulina* samples and further fractions on the expression of apoptotic genes of HT-29 cells and antioxidant genes of RAW264.7 were investigated by the Quantitative Polymerase Chain Reaction (qPCR) technique. Specific TaqMan® probes of Bax, Bcl-2, caspase-3, Nrf2, HO-1, NQO1, Gclc, Srxn1, and lastly β-actin as endogenous control were used for analysis. The treatment doses of the fermented *Spirulina* samples and further fractions were chosen as IC_{50} and $IC_{50}x2$ values. The effects of these products on mRNA expression levels were evaluated by the comparative ΔΔCt method.

3.2.6. Protein Expression Analysis

3.2.7. Protein Extraction and SDS-PAGE

After the cells are treated with fermented *Spirulina* products for 4 h, Cytoplasmic, and nuclear proteins were isolated from RAW264.7 cells by using NE-PER™ Nuclear and

Cytoplasmic Extraction Kit according to the specifications of the kit. The concentration of protein lysates was determined via BCA assay with Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions.

Proteins were denatured in the water bath for 10 mins at 70 °C before loading to gel with the addition of Pierce™ LDS Sample Buffer and Bolt™ Sample Reducing Agent. 4% stacking and 12% separating gels were prepared and protein samples were loaded evenly. Proteins were separated based on their sizes with electrical force.

3.2.8. Western Blot

The gel was placed into a sandwich-like system that includes sponges, Whatman papers, gel, and nitrocellulose membrane to blot proteins on the gel to membrane. The preparation of the system proceeded in cold transfer buffer (10% methanol, 192 mM glycine, 25 mM Tris) and also the tank was filled with transfer buffer. Blotting proceeded for 1 h with a constant voltage (10 V). After blotting, the membrane was incubated in a blocking solution (5% non-fat dry milk in TBST) for 1 h. The membrane was placed in Nrf-2 (1:100) and β-actin (1:200) antibody solutions, separately, and incubated overnight at 4 °C. Primary antibody was removed and the membrane was incubated in proper secondary antibody (1:1000) for 2 h, at room temperature. Lastly, the membrane was subjected to SuperSignal™ West Pico PLUS Chemiluminescent substrate for 10 mins at dark. The membrane was scanned by the C-DiGit® Blot Scanner and densitometric analysis was carried out by using Image Studio Digits Software 5.0.
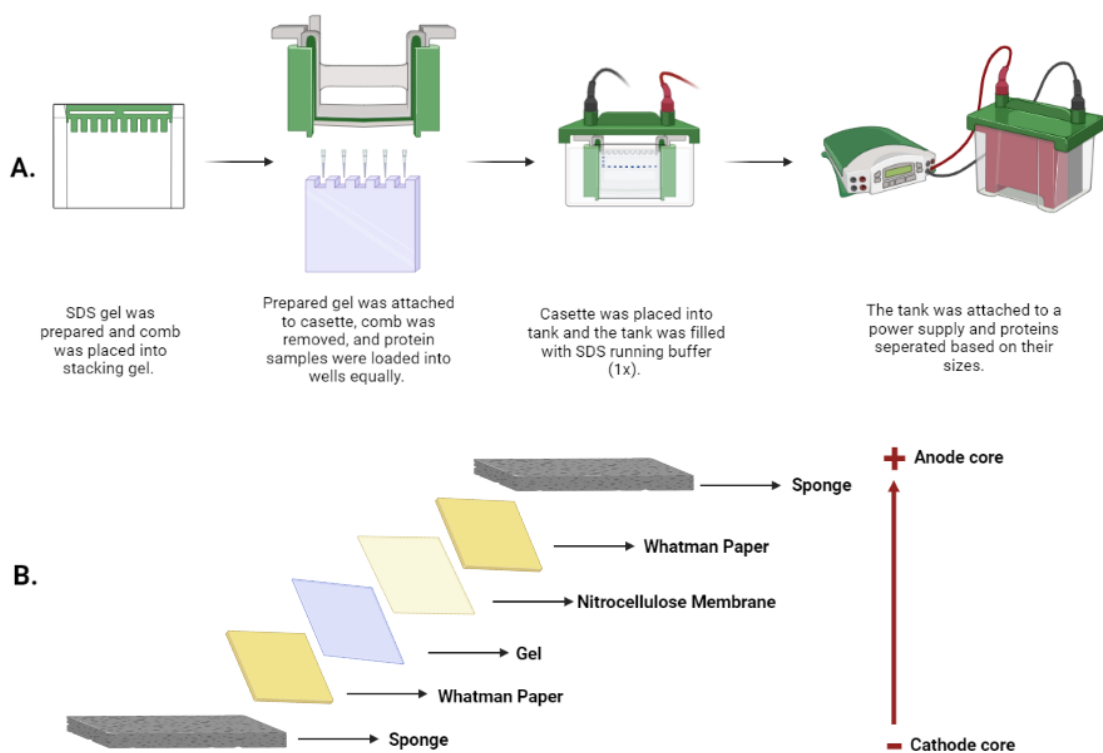
Figure 4. Experimental steps of SDS-PAGE (A) and sandwich-like system of Western Blot (B).

3.2.9. Peptide Fractionation by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

Further purification of bioactive 5-10 kDa UF fraction of FS protein hydrolysate was performed by SHIMADZU semi-preparative high-performance liquid chromatography (HPLC) instrument. Two different mobile phases were used including water containing 0.1% trifluoroacetic acid (TFA) (mobile phase A), and acetonitrile containing 0.1% trifluoroacetic acid (TFA) (mobile phase B). The sample and solvents were filtered through 0.45, and 0.22 μm membranes, respectively, before applying them to the column. After the preparation of the sample, it was applied to the Teknokroma Europa Peptide C18 column. Fractions were injected (500 μL) and, eluted at a 2.5 mL/min flow rate by a linear gradient of 10-30% mobile phase B for 40 minutes of the total analysis. *Spirulina* peptide fractions were detected via a deuterium lamp at 220 nm and collected via the fraction collector. Solvents of collected fractions were evaporated by using BUCHI Interface I-300 Rotary Evaporator System.

3.2.10. Statistical Analysis

All statistical analyses were carried out via GraphPad Prism 8 to determine the significance of the difference between obtained data. Technical and biological replicated experiments were performed and results were represented as the standard error of mean ± (SEM). For comparison between control and treatment groups, One-way analysis of variance (ANOVA) was used.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Results

In this study, FS products were investigated in terms of their NO suppressive capacity and their effects on cell viability. Firstly, FS products produced by three different microbial fermentations (by *Lactobacillus helveticus*, *Kluyveromyces marxianus*, and in the combination of both microorganisms) were tested and one FS product was chosen according to its promising bioactive properties. With the chosen microbial group, different batch operational bioreactor productions were performed in the 3-L bioreactor by our collaborative research group. Among these products, one with the most promising bioactive properties was used for UF fractionations (>2 kDa, 2-5 kDa, 5-10 kDa). Moreover, each UF fraction was tested and chosen fraction was further fractioned into its fractions by reverse phase high-performance liquid chromatography (RP-HPLC). In this bioactivity-oriented fractionation process, the difference between FS products and their unfermented counterparts was investigated. To finalize the study, the effects of promising products on antioxidant and anticancer genes were evaluated. Additionally, the effects of these products on the activation of Nrf-2 were presented by using the Western Blot technique.

4.1.1. Bioactivity of Optimization Fermentation Hydrosylates

4.1.2. Effects of Optimization Fermentation Hydrosylates on Cell Viability

The effects of fermented *Spirulina platensis* by different organisms, *Kluyveromyces marxianus* (KM), *Lactobacillus helveticus* (LH), in the combination of both *Kluyveromyces marxianus* and *Lactobacillus helveticus* (mix), on the viability of HUVEC, RAW 264.7, and HT-29 cell lines were investigated by SRB cytotoxicity assay. Cell lines were treated with fermented (F) products and their unfermented counterparts (C) for 48 h at doses of 30, 60, 90 μg/mL in terms of protein concentrations. KM-F product did not decrease the viability of healthy or cancer cell lines by 50%. Although, the maximum inhibition of KM-F was approximately 36% on HT-29 colon cancer cells while on healthy cells maximum inhibition was nearly 26% (Fig. 5A). LH-F product did not show any inhibitory or promotive effects on the viability of cell lines similar to LH-C product (Fig. 5B). Moreover, mix-F increased

the viability of RAW 264.7 by 20% at all doses whereas mix-C did not show any significant effects. Yet, mix-F did not inhibit the viability of colon cancer cells, while mix-C decreased the cell viability to 47% which is an unexpected result (Fig. 5C).
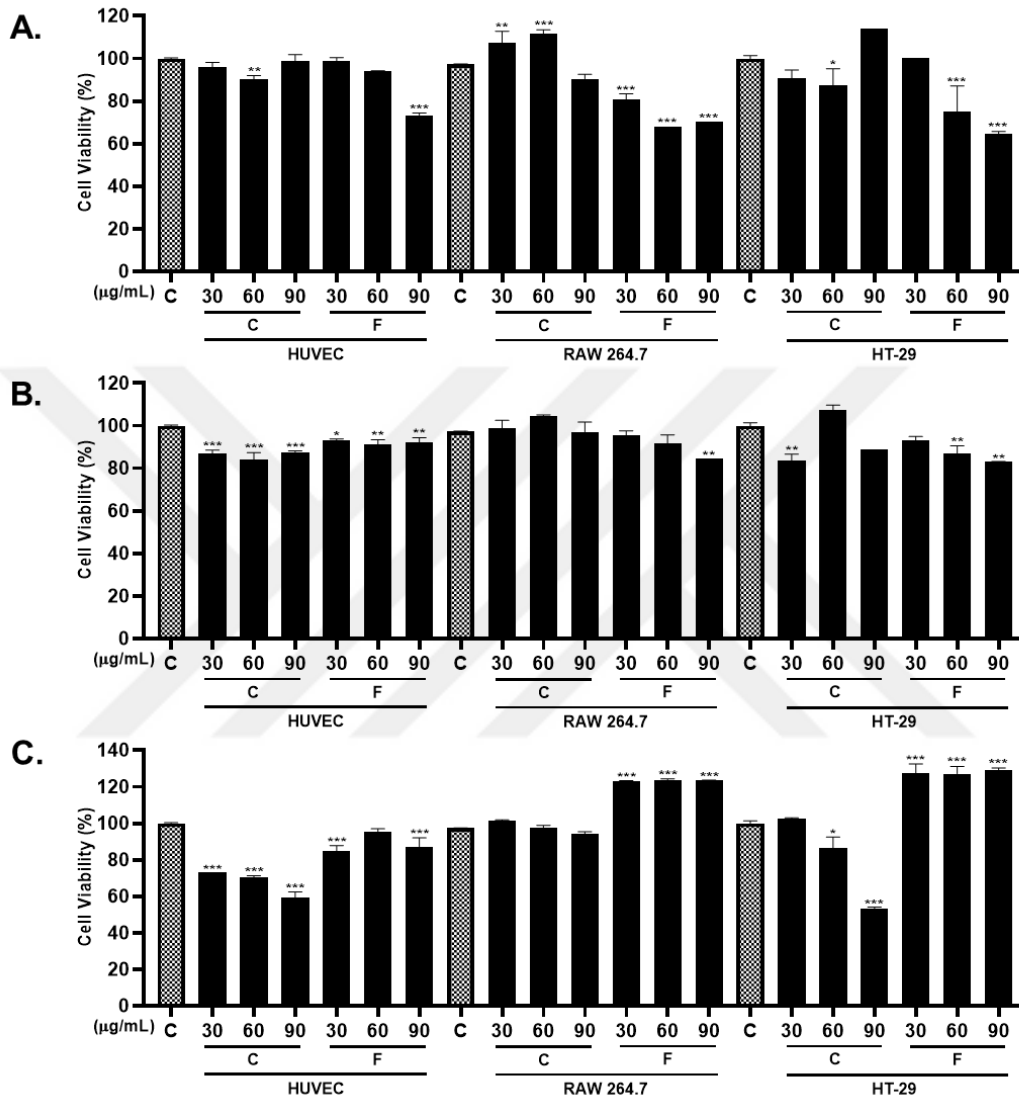

Figure 5. Cytotoxicities of fermented (F) and unfermented (C) *Spirulina platensis* on HUVEC, RAW 264.7, and HT-29 cell lines at 30-60-90 μg/mL doses in terms of protein concentration. *Spirulina platensis* fermented by *Kluyveromyces marxianus* (A), *Lactobacillus helveticus* (B), and mixed culture of *Kluyveromyces marxianus* and *Lactobacillus helveticus* (C). C: Control. *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.3. Evaluation of the Effects of Optimization Fermentation Hydrosylates on LPS-induced NO Secretion

The effects of fermented *Spirulina platensis* by different organisms, *Kluyveromyces marxianus* (KM), *Lactobacillus helveticus* (LH), mixed culture of *Kluyveromyces marxianus* and *Lactobacillus helveticus* (mix), on the LPS-induced NO production of RAW 264.7 cells were elucidated by using Griess assay. To determine if the suppression of NO release was caused by cell death, data were normalized by using MTT cytotoxicity data. As compared to the LPS-free control group, LPS treatment induced NO secretion of macrophages. KM-fermented product suppressed the NO production at 60 and 90 μg/mL doses by approximately 20% and 10%, respectively. Similar to this, the LH-fermented product also showed a suppressive effect on NO production approximately by 20% at 30 and 90 μg/mL doses. Yet it was observed that all unfermented products increased NO release, compared to the LPS-induced control group, up to nearly 230%, 177%, and 227% for KM-C, LH-C, and mix-C, respectively, at the lowest dose-treated (30 μg/mL). However, the mixed culture-fermented product showed a distinctive potency i.e. 89-95% suppressive effect on LPS-induced NO production for 30-90 μg/mL doses (Fig. 6).
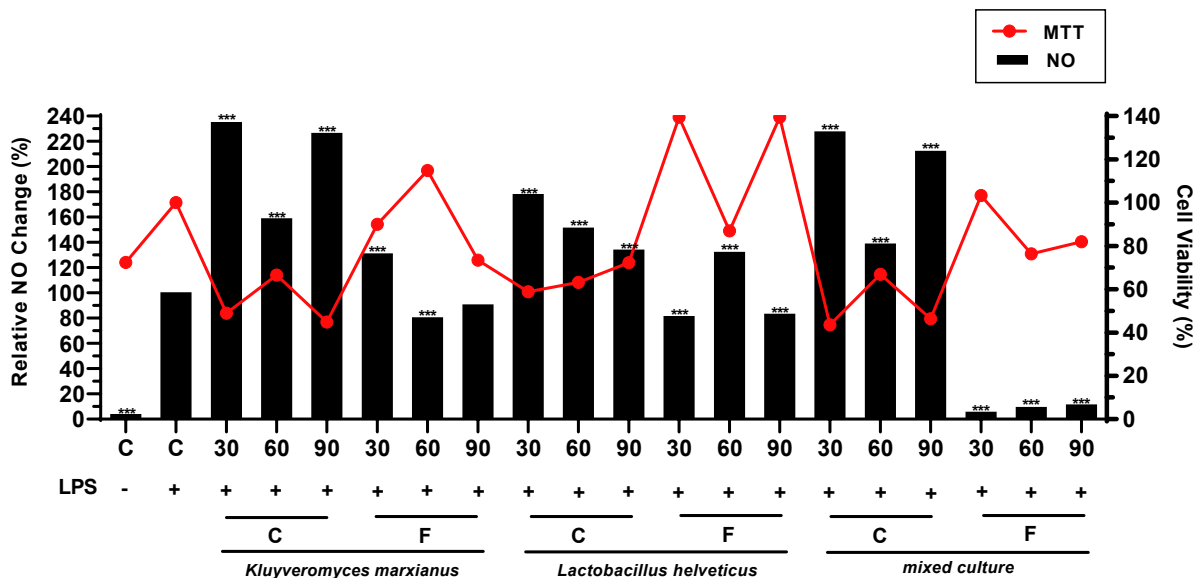

Figure 6. Effects of fermented (F) and unfermented (C) *Spirulina platensis* on NO secretion and viability of LPS-induced RAW 264.7 cells. C: Control, C: Control (Only LPS). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.4. Bioactivity of Bioreactor Fermentation Hydrolysates

4.1.5. Screening of Anticancer Effects of Bioreactor Fermentation Hydrosylates by SRB Assay

Spirulina Platensis hydrolysates as a result of fermentation by mixed culture of *Kluyveromyces marxianus* and *Lactobacillus helveticus* on bioreactor level performed with different operational conditions were investigated in terms of their anti-cancer activities on HT-29 cells (Fig. 7A) and additionally on healthy cell line (HUVEC) (Fig. 7B) by SRB assay at 48 h. Also, the unfermented control groups were tested to determine the difference between the activities of fermented (M2, M4, M6, M8) and their unfermented counterparts (M1, M3, M5, M7). Fermented and unfermented products were treated at 100, 500, and 1000 µg/mL doses for the screening. From the treated products, M2 inhibited the cell viability by 50% at the highest dose (1000 µg/mL) while other doses had no inhibitory effects. Favorably, M6 decreased the cell viability in a dose-dependent manner whereas its unfermented control group did not show an inhibitory effect. The viability of colon cancer cells decreased by nearly 30%, 60%, and 72% at the doses of 100, 500, and 1000 µg/mL, respectively (Fig. 7). In the light of data presented in Figure 7, M6 was chosen for further assays.
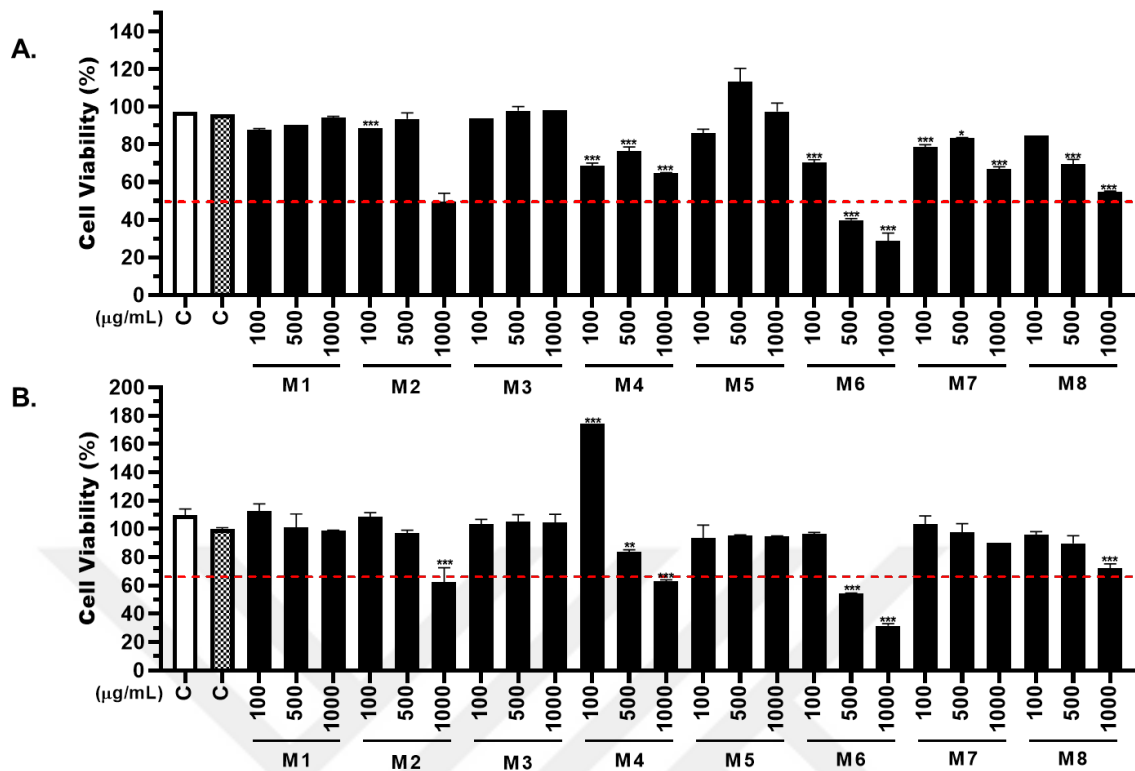
Figure 7. Effects of fermented (M2, M4, M6, M8) and unfermented (M1, M3, M5, M7) *Spirulina platensis* bioreactor products on the viability of HT-29 (A), and HUVEC (B) cell lines. C: Control, C: Control (Only solvent). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.6. Time Optimization and IC_{50} Determinations of M6

The optimal time interval of treatment for IC_{50} screening assays was determined by SRB assays with treatment durations of 24, 48, and 72 h on HT-29 and HUVEC cell lines. 10, 50, 100, 250, 500, and 1000 µg/mL doses of M6 and M5 were treated and IC_{50} values were determined for each time interval considered. For all time durations, a dose-dependent inhibition pattern was observed for 250, 500, and 1000 µg/mL doses of M6. However, the unfermented product did not decrease the cell viability under 50% except for the 500 and 1000 µg/mL doses of 72 h treatment. Even though at 72 h, M6 decreased the cell viability approximately to 13%, and 3% where M5 decreased to 41%, and 45% for 500 and 1000 µg/mL doses, respectively (Fig 8.C). Additionally, M6 showed more cytotoxicity to HT-29 cells as compared to the healthy cell line. Considering IC_{50} values, 48 h treatment of M6 was observed as the most efficient compared to 24 h (IC_{50}: 332.6 µg/mL), and 72 h (IC_{50}: 282.8)

μg/mL) treatment durations with the IC_{50} value of 238.8 μg/mL (Table 5). Moreover, the selectivity index (SI) of M6 was determined as 3.1 at 48 h.
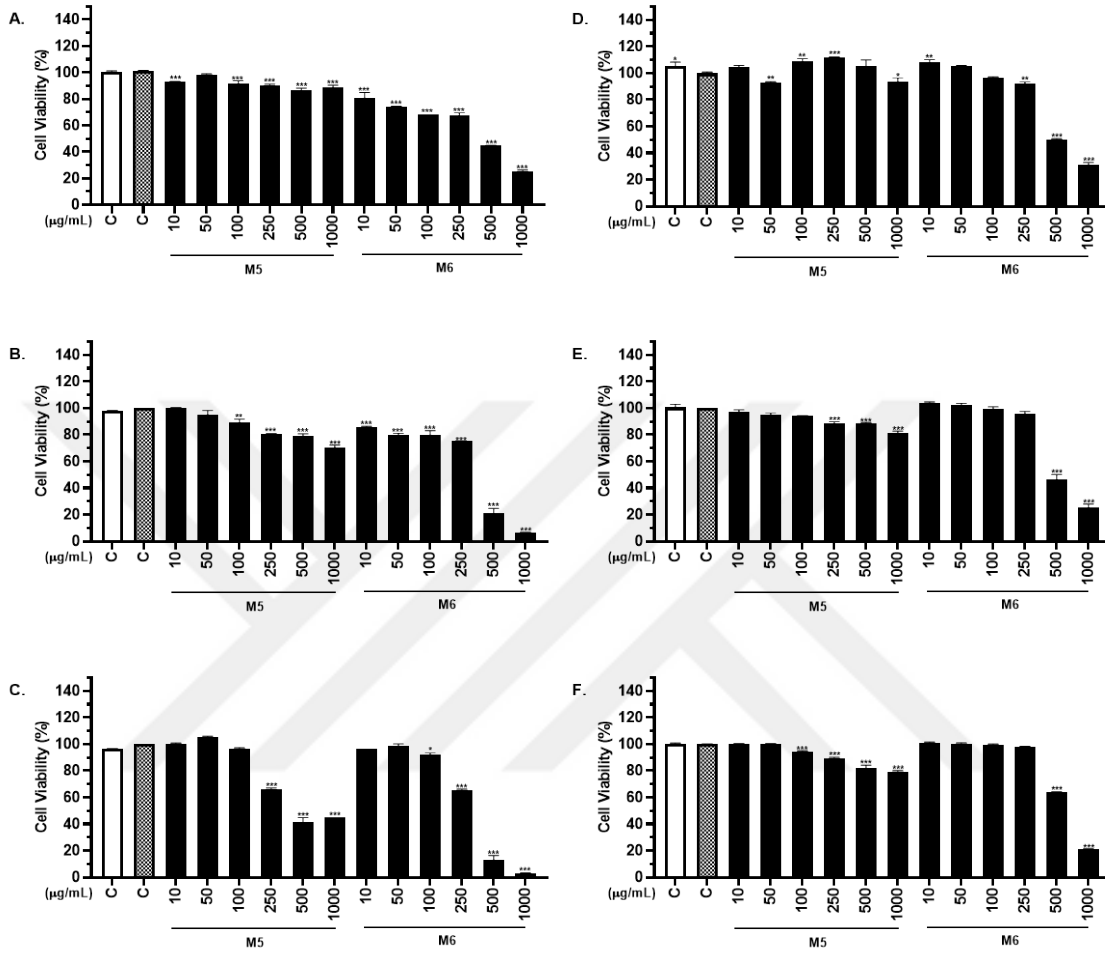

Figure 8. Effects of fermented (M6) and unfermented (M5) *Spirulina platensis* bioreactor products on the viability of HT-29 (A, B, C), and HUVEC (D, E, F) cell lines at 24 h (A, D), 48 h (B, E) and, 72 h (C, F). C: Control, C: Control (Only solvent). *p < 0.02, **p < 0.005, ***p < 0.001

Table 5

IC_{50} values of fermented (M6) and unfermented (M5) *Spirulina platensis* bioreactor products at 24, 48, and 72 h for HT-29, and HUVEC cell lines.

Cell Line	Incubation	24 h	48 h	72 h	Cell Line	Incubation	24 h	48 h	72 h
	Products	IC_{50}	IC_{50}	IC_{50}		Products	IC_{50}	IC_{50}	IC_{50}
HT-29	M5	5023	1863	631.8	HUVEC	M5	73701	3636	2929
	M6	332.6	238.8	282.8		M6	743.4	759.4	905.5

4.1.7. Evaluation of Anticancer Effects of M6 on MCF-7 and PC-3 Cell Lines by SRB Assay

After the determination of the optimal time interval for SRB assay and evaluation of anticancer effects of M6 on HT-29 cell line, for the further estimation of hydrolysates anticancer activities on MCF-7 and PC-3 cell lines IC_{50} screening performed by SRB assay at 48 h. The same doses as the treatment of HT-29, and HUVEC (10, 50, 100, 250, 500, and 1000 μg/mL) were used.

The only cytotoxic dose of M6 on breast cancer cell line determined as 1000 μg/mL, decreased cell viability by 66% where none of the doses of M5 affected the cell viability (Fig. 9A). Differently, M6 decreased the viability of prostate cancer cells approximately by 80% (at 1000 μg/mL) in a dose-dependent manner and it was also observed that high doses of the unfermented group showed a promoting effect by increasing the viability of PC-3 cells nearly up to 140% (Fig. 9B). Moreover, IC_{50} values of M6 estimated as 1454, and 514.4 μg/mL in MCF-7, and PC-3 cell lines, respectively.
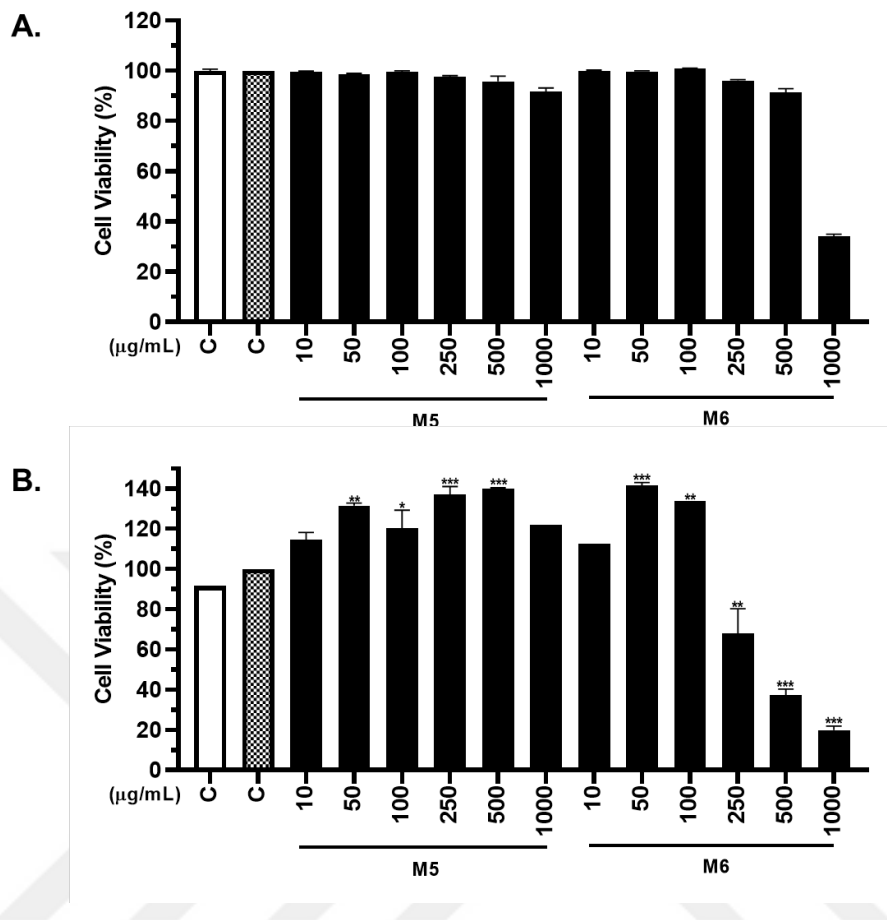
Figure 9. Effects of fermented (M6) and unfermented (M5) *Spirulina platensis* bioreactor products on the viability of MCF-7 (A), and PC-3 (B) cell lines at 48 h. C: Control, C: Control (Only solvent). *p < 0.02, **p < 0.005, ***p < 0.001

4.1.8. Evaluation of the Effects of M6 on LPS-induced NO Secretion

Suppressive effects of M6 on LPS-induced NO secretion of macrophages were evaluated by Griess assay and obtained data were normalized by using MTT assay results. Cells were subjected to 100, 500, and 1000 μg/mL doses of M5, and M6. 500 and 1000 μg/mL doses of M6 showed considerable potency by suppressing the NO release to 24%, and 48%, respectively. In view of the fact that 1000 μg/mL dose of M6 decreased the number of viable cells by 77%, 500 μg/mL may be indicated as a safe dose for suppressing the NO release without showing any cytotoxic effects to cells (Fig. 10).
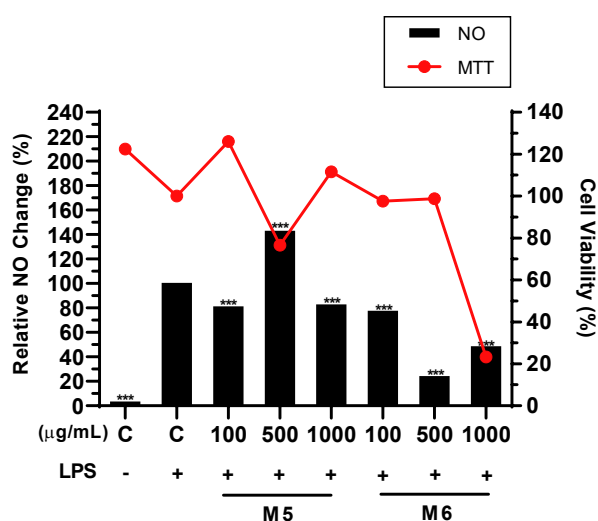
Figure 10. Effects of fermented (M6) and unfermented (M5) *Spirulina platensis* on NO secretion and viability of LPS-induced RAW 264.7 cells. C: Control, C: Control (Only LPS). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.9. Bioactivity of three different UF Fractions of M6

4.1.10. IC$_{50}$ Determinations of M6 UF Fractions

IC$_{50}$ screenings for >2, 2-5, and 5-10 kDA UF fractions were accomplished by SRB assays at 48 h for HT-29 and HUVEC cell lines. 10, 50, 100, 250, 500, and 1000 µg/mL doses of UF fractions were treated and IC$_{50}$ values were determined for each UF fraction of M5, and M6. Among all three UF fractions treated, two of them (2-5 kDa, and 5-10 kDa) were able to decrease colon cancer cell viability under 50% yet the inhibitory effect of these fractions was not in a dose-dependent manner. For >2 kDa, and 2-5 kDa UF fractions; IC$_{50}$ values were determined as 258.6 (M5), and 513.5 (M6); 116.4 (M5), and 252 (M6) µg/mL, respectively. On the other hand, IC$_{50}$ values for 5-10 kDa UF fractions were 636.7 (M5), and 248.3 (M6) µg/mL, indicating that 5-10 kDa fraction have more potency to show an inhibitory effect on the viability of colon cancer cells as compared to the unfermented 5-10 kDa fraction (Fig. 11B). In sum, 5-10 kDa UF fraction of M6 is the only fraction with the higher potential anti-cancer activity compared to the unfermented (M5) UF fractions considering the calculated IC$_{50}$ values no cytotoxicity to HUVEC cells were demonstrated (Fig. 11A). Favorably, SI index (32.4) for 5-10 fraction of M6 implies that it showed selective cytotoxicity for colon cancer cells.
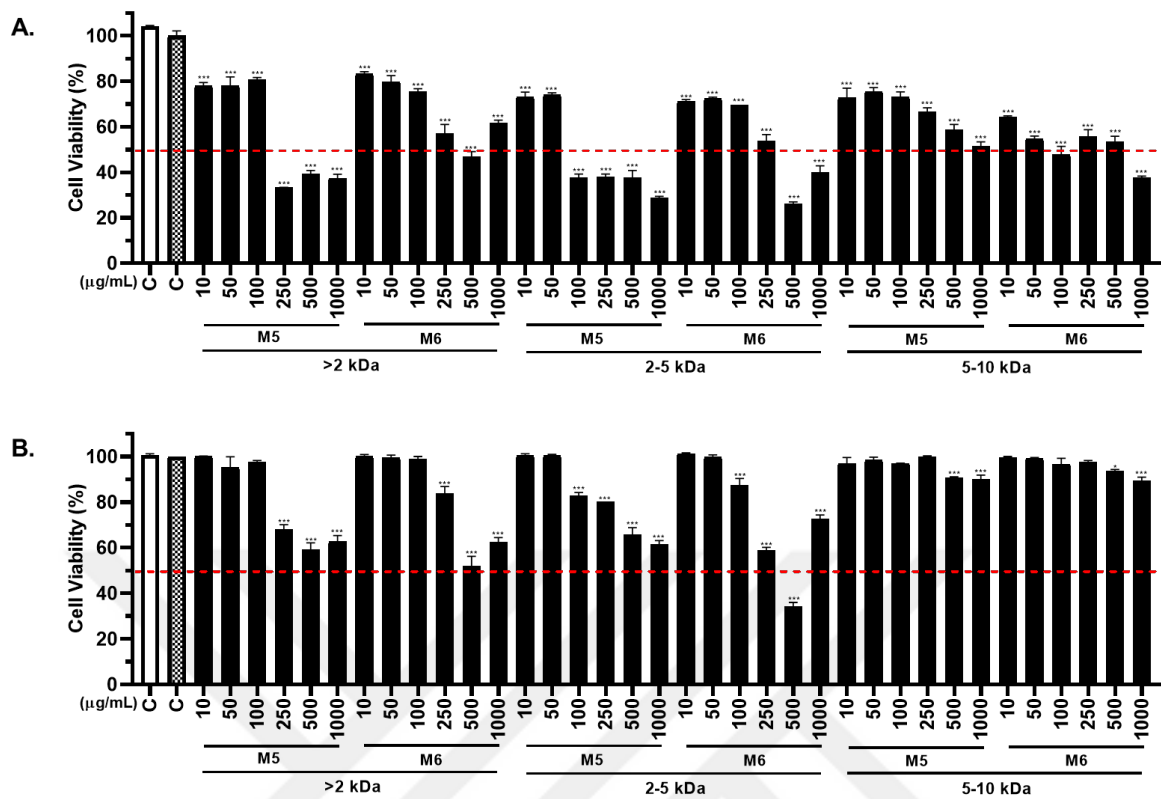
Figure 11. Effects of fermented (M6) and unfermented (M5) UF fractions on the viability of HT-29 (A), and HUVEC (B) cell lines at 48 h. C: Control, C: Control (Only solvent). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.11. Evaluation of the Effects of M6 UF Fractions on LPS-induced NO Secretion

The potency of M6 UF fractions for suppressing the LPS-induced NO secretion of macrophages was investigated by Griess assay and obtained data were normalized by using MTT assay results. In the light of previous data, since the determined safe dose of M6 was 500 μg/mL, treated doses were chosen as 50, 100, and 200 μg/mL. Interestingly, UF fractions did not show a significant suppressive effect on the NO release, in fact, 50 μg/mL of 2-5 kDa, and 100 μg/mL of 5-10 kDa induced the released NO level nearly by 150% (Fig. 12).
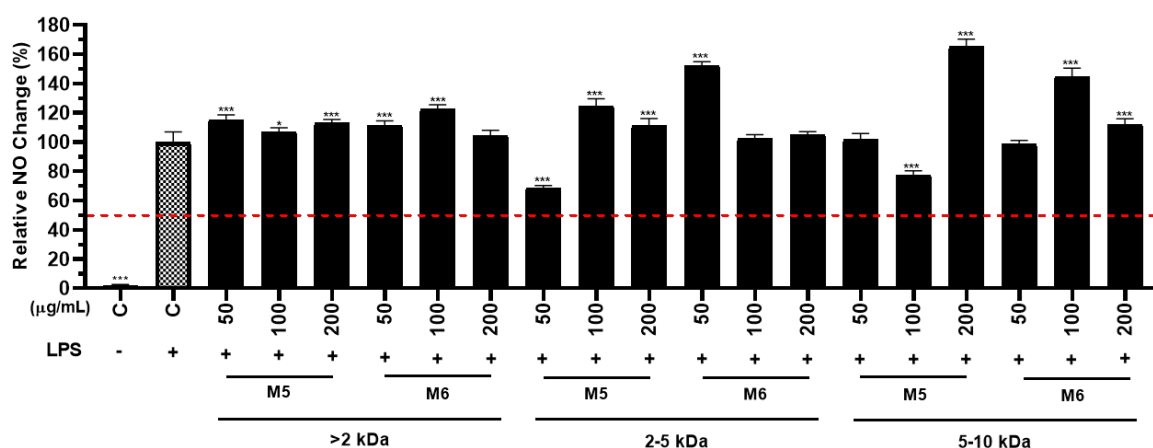
Figure 12. Effects of fermented (M6) and unfermented (M5) UF fractions on NO secretion of LPS-induced RAW 264.7 cells. C: Control, C: Control (Only LPS). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.12. RP-HPLC Method Optimization and Collection of Peptide Fractions

Among the three UF fractions, 5-10 kDa demonstrated selective cytotoxicity to HT-29 cells. Thus, it was selected for further separation into peptide fractions to investigate the bioactivity and establish if the bioactivity is originate in peptides. For this purpose, a RP-HPLC procedure was optimized by changing variable parameters, including analysis duration (20-60 min), the gradient of mobile phases, and flow rate (1.00-5.00 mL/min). Optimization of the method was completed in analytical mode, and then the collection of peptide fractions was carried out. Used mobile phases were water containing 0.1% trifluoroacetic acid (TFA) (mobile phase A), and acetonitrile containing 0.1% trifluoroacetic acid (TFA) (mobile phase B).

Spectrums of some of the methods tested and optimized method were demonstrated in Figure 13. In detail, peaks were detected via the linear gradient of 80-40% mobile phase B was tested with the flow rate of 2.5 mL/min in a 40 min analysis (Fig. 13A). Subsequently, the same analysis duration, and flow rate with the linear gradient of 25-75% mobile phase B (Fig. 13B) and peak intervals were nearly same in both gradients. Another attempt was proceeded by decreasing the ratio of mobile phase B and increasing the analysis duration; with a linear gradient of 0.1-20% mobile phase B in 60 min (Fig. 13C). Better peak resolution was observed in 40 min of total analysis and low mobile phase B ratio when the obtained

spectrums compared. Four peptide fractions were collected (FR1, FR2, FR3, and FR4) at a 2.5 mL/min flow rate by a linear gradient of 10-30% mobile phase B for 40 minutes of the total analysis (Fig. 13D). During the whole process, peptide fractions were collected at 220 nm wavelength.
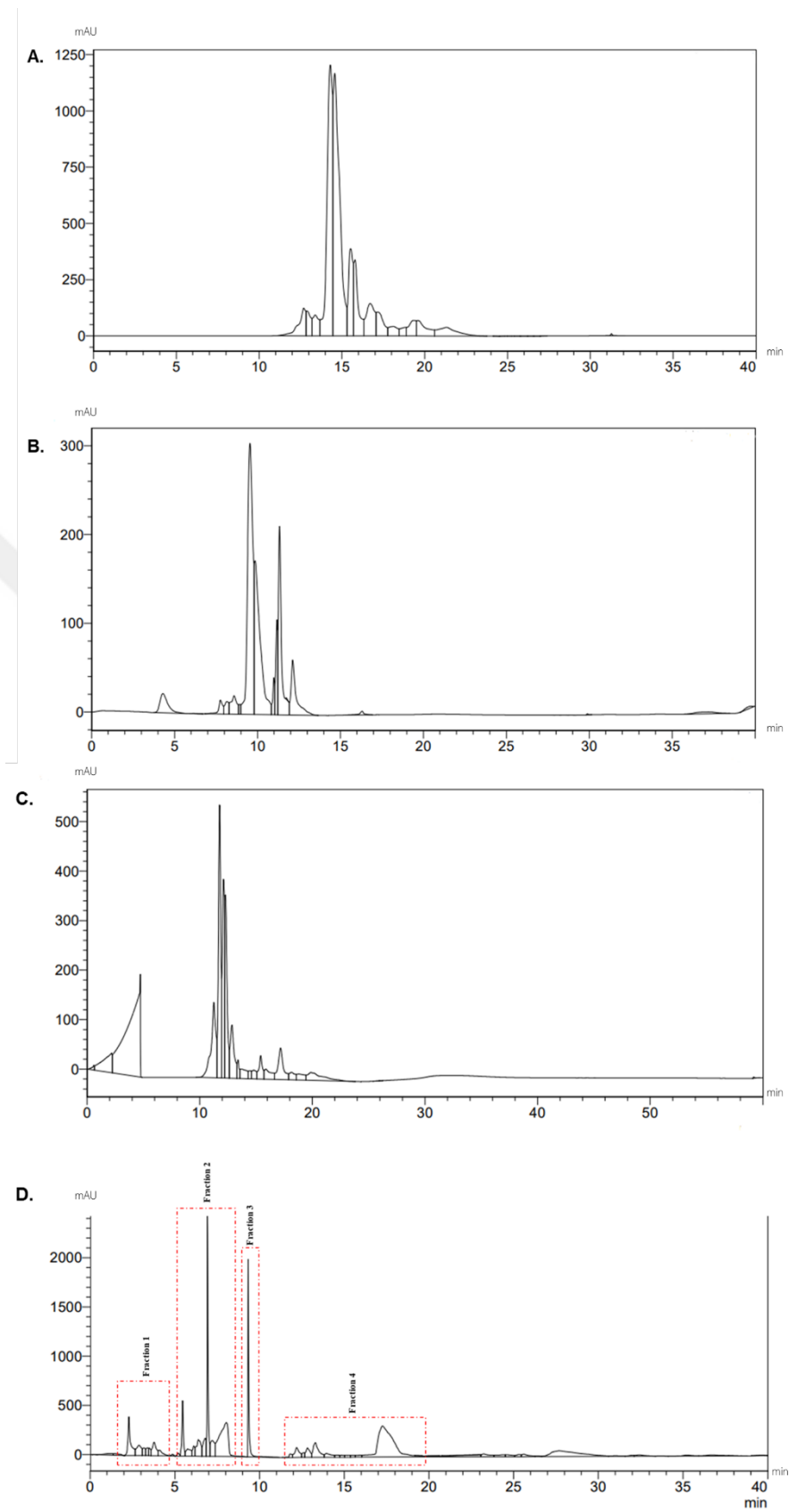
Figure 13. HPLC spectrums of optimization process (A, B, C), optimized method, and collected peptide fractions (D).

4.1.13. Bioactivity of Fermented *Spirulina* Peptide Fractions

4.1.14. Screening of Anticancer Effects of Fermented *Spirulina* Peptide Fractions by SRB Assay

Fermented *Spirulina* peptide fractions were investigated in terms of their anti-cancer activities on HT-29 cells (Fig. 14A) and additionally on HUVEC (Fig. 14B) to demonstrate the selective cytotoxic effects of the peptide fractions by SRB assay at 48 h. Peptide fractions were treated to cells in a wide dose range (100, 500, and 1000 μg/mL) for a general estimation of their activities. FR3 and FR4 did not affect the viability of healthy or colon cancer cells. On the other hand, the 1000 μg/mL dose of FR2 decreased cell viability approximately by 47% (Fig. 14A). Yet, FR1 showed higher potency compared to FR2 by demonstrating cytotoxicity nearly above 80% at both 500, and 1000 μg/mL doses (Fig. 14A) without implying a negative effect on healthy cells (Fig. 14B).
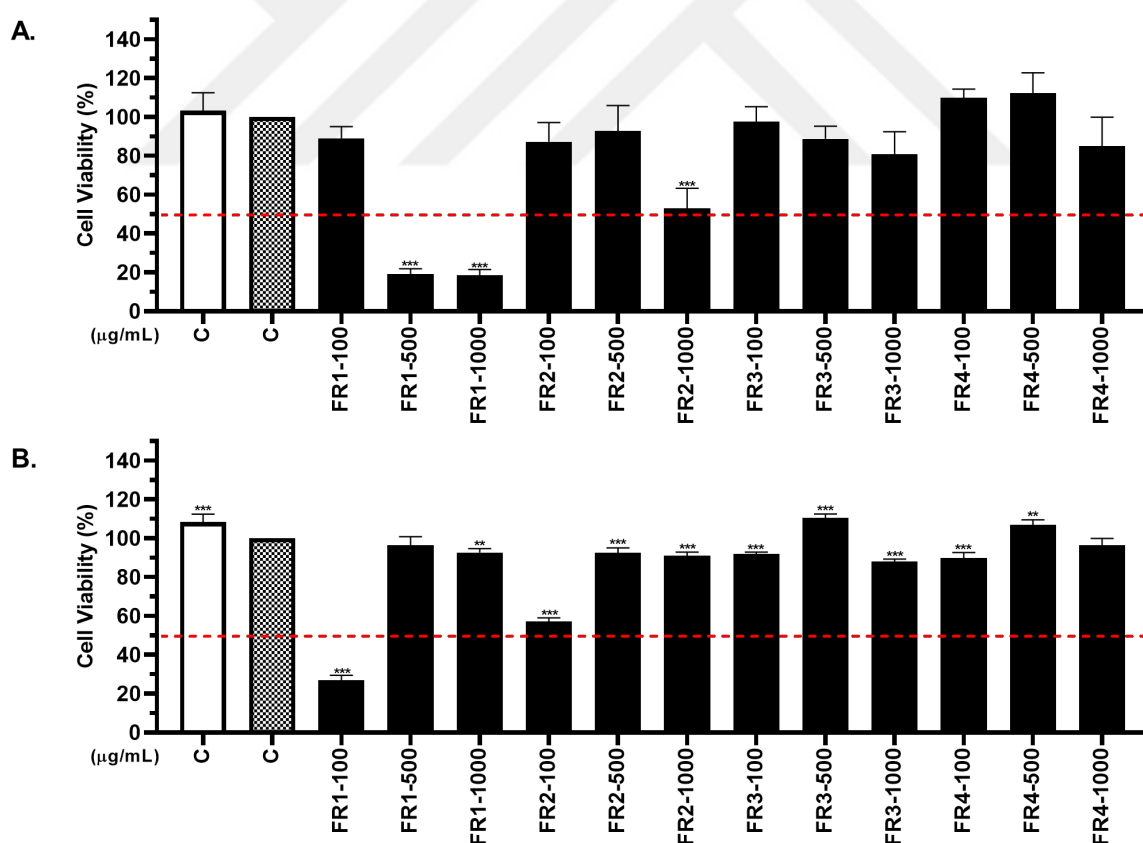

Figure 14. Effects of fermented *Spirulina* peptide fractions on the viability of HT-29 (A), and HUVEC (B) cell lines at 48 h. C: Control, C: Control (Only solvent). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.15. IC$_{50}$ Determinations of Fermented *Spirulina* Peptide Fractions FR1 and FR2

Subsequent to the evaluation of active fractions in terms of potential anti-cancer properties, IC50 screening for FR1 and FR2 was accomplished via SRB assay. 50, 100, 250, 500, and 1000 µg/mL doses of peptide fractions were treated to cells for 48 h. Where FR2 did not indicate cytotoxicity above 50%, FR1 selectively (SI: 5.5) decreased the cell viability of colon cancer cells nearly by 90% at 1000 µg/mL dose, in a dose-dependent manner. IC$_{50}$ values for FR1, and FR2 were calculated as 250.4, and 728, respectively.
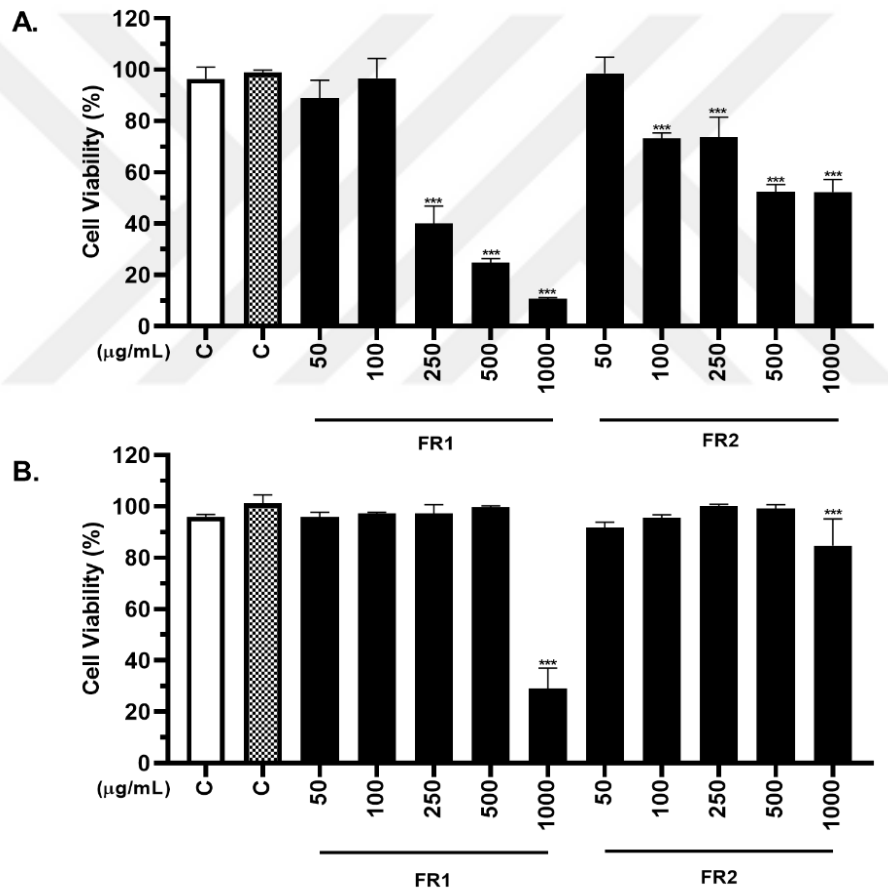

Figure 15. Effects of FR1, and FR2 on the viability of HT-29 (A), and HUVEC (B) cell lines at 48 h. C: Control, C: Control (Only solvent). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.16. Evaluation of the Effects of Fermented *Spirulina* Peptide Fractions on LPS-induced NO Secretion

Potential suppressive effects of fermented *Spirulina* peptide fractions on the NO secretion of macrophages stimulated by LPS treatment was established by Griess assay and obtained data were normalized by using MTT assay results. None of the peptide fractions suppressed the released NO level by 50%. The maximum positive effect was observed at 1000 μg/mL dose of FR4, suppressing NO release nearly 33% (Fig. 16). Conspicuously, peptide fractions which were demonstrated to have potential anticancer effects, promoted the secreted NO amount nearly as twice, compared to the LPS-induced control group.
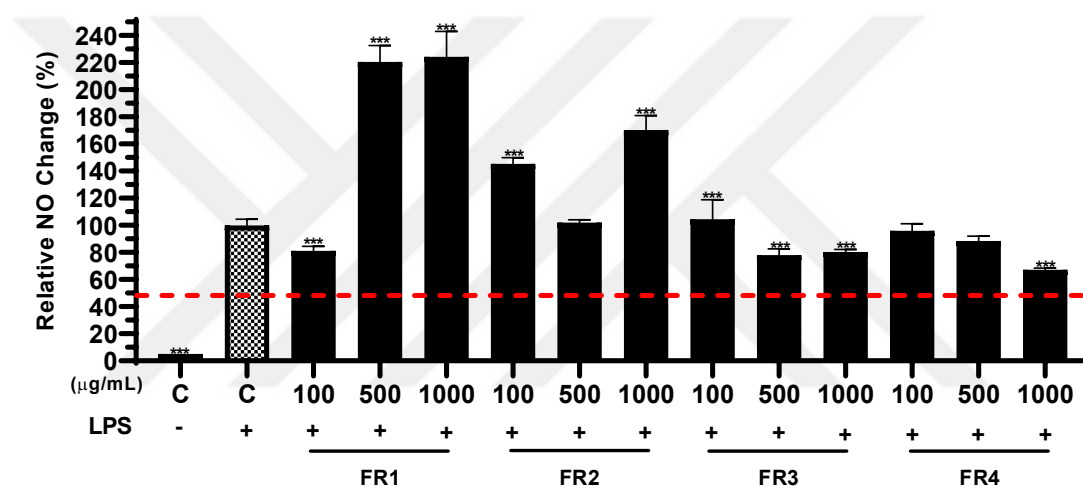

Figure 16. Effects of fermented *Spirulina* peptide fractions on NO secretion of LPS-induced RAW 264.7 cells. C: Control, C: Control (Only LPS). *p < 0.02, **p < 0.005, ***p < 0.001.

4.1.17. Mechanism Studies of Active Hydrolyzates and Further Fractions

4.1.18. Gene Expression Analysis

As previously demonstrated, the following products were distinguished among all investigated products by indicating selective cytotoxicity on colon cancer cells: M6, 5-10 kDa UF fraction of M6, and further purified peptide fractions of 5-10 kDa UF product FR1, and FR2. Due to their potency to carry out anticancer activities, the effects of these products on pro-apoptotic gene caspase-3 were determined by qPCR analysis and quantified by the comparative ΔΔCt method. Doxorubicin was used as a positive control and cells were subjected to two different doses (IC_{50} values and twofold IC_{50} values) of M6, 5-10 kDa UF fraction of M6, FR1, and FR2 for 48 h before the RNA isolation. B-actin was used as

endogenous control. Any of the products upregulated the expression of Caspase-3 on the mRNA level where positive control caused a significant upregulation.
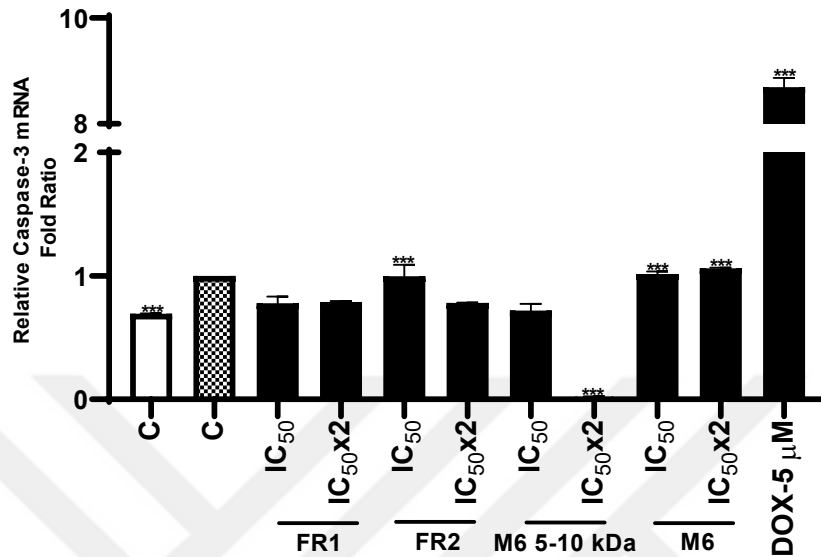

Figure 17. Effects of FR1, FR2, M6 5-10 kDa, and M6 to the expression of Caspase-3 gene. C: Control, C: Control (Only Solvent), DOX: Positive Control. (Doses: 250.4, 500.8 µg/mL for FR1; 728, 1456 µg/mL for FR2; 248.3, 486.6 µg/mL for M6 5-10 kDa; 238.8, 477.6 µg/mL for M6.) *$p < 0.02$, **$p < 0.005$, ***$p < 0.001$.

The mix-fermented hydrolysate of *Spirulina* and M6 demonstrated suppressive effects on LPS-induced NO release of macrophages so the potential anti-inflammatory effects of these products were investigated by analyzing the related genes (IL-1β, and TNF-α) expressions on mRNA level and by protein quantification studies via IL-1β, and TNF-α ELISA assays (Appendix-Figure 1, Figure 2). The inflammation model was constituted by the stimulation of RAW 264.7 via LPS treatment for 24 h and products were treated to cells simultaneously. At the end of 24 h incubation, the RNA of the samples was isolated. Since the results of mechanism studies were not supporting the previous studies, data are not given in this section.

Promising agents due to cytotoxicity and NO suppressive potential results were investigated for their indirect antioxidant activities at 24 h by using macrophages. Effects of FR1, FR2, M6 5-10 kDa, and M6 preliminarily investigated on the expression of Nrf-2 since

it carries out a role as a mediator for antioxidant and detoxification genes. 500 μg/mL doses of M6 5-10 kDa, and M6 significantly enhanced the gene expression of Nrf-2 approximately 3 fold at the mRNA level (Fig. 18).
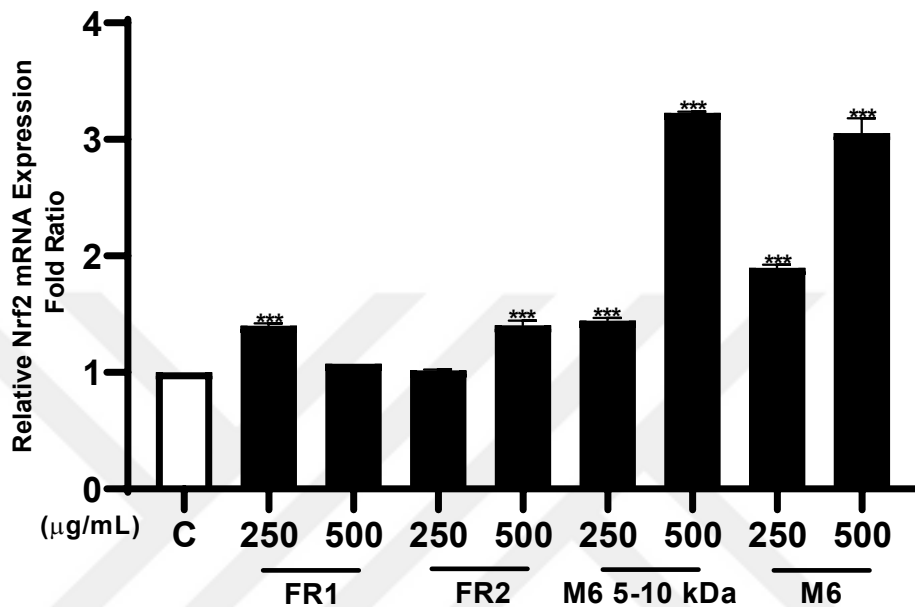

Figure 18. Effects of FR1, FR2, M6 5-10 kDa, and M6 to the expression of Nrf-2 gene. C: Control. $*p < 0.02$, $**p < 0.005$, $***p < 0.001$.

Gene expression analysis carried on with antioxidant and detoxification genes, assuming that if tested products were upregulated the Nrf-2 expression; they might affect the relevant genes in a similar pattern. FR1, FR2, M6 5-10 kDa, and M6 were treated to macrophages for 24 before the RNA isolation. Effects of these products on expressions of detoxification genes NQO1, Gclc, and antioxidant genes HO-1, and Srxn1 were determined by qPCR analysis and quantified by the comparative ΔΔCt method.

Similar to the Nrf-2 gene expression results, M6 5-10 kDa, and M6 implicated significant and distinctive effects. 250, and 500 μg/mL doses of M6 increased the expressions of HO-1 by 4.6, and 11.9 fold (Fig. 19A); NQO1 by 2.6, and 8.8 fold (Fig. 19B); Srnx1 by 33, and 116 fold (Fig. 19C); Gclc by 4.3, and 4.7 fold (Fig. 19D), respectively. In the case of 250, and 500 μg/mL doses of 5-10 kDa M6, they increased the expressions of

HO-1 by 3.3 (Fig. 19A), and 4.2 fold; NQO1 by 1.2 (Fig. 19B), and 1.9 fold; Srnx1 by 6.9, and 30.5 fold (Fig. 19C); Gclc by 1.9, and 4.4 fold (Fig. 19D), respectively.
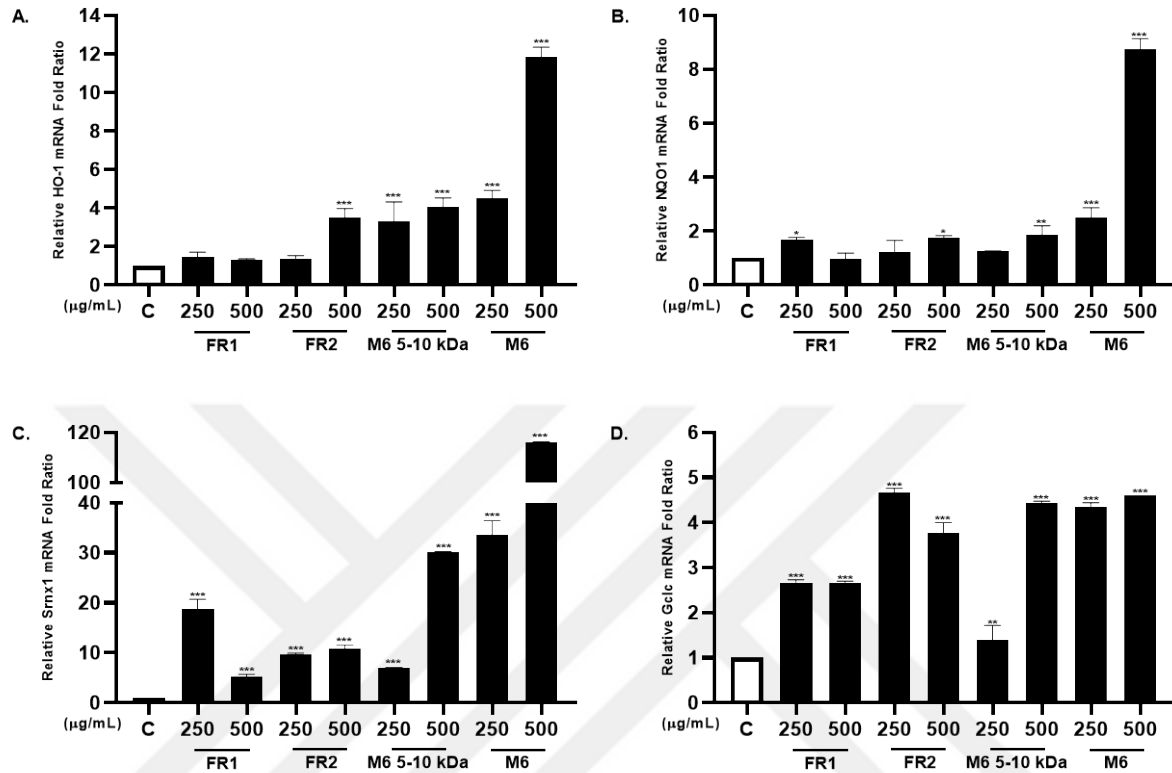

Figure 19. Effects of FR1, FR2, M6 5-10 kDa, and M6 on the expressions of HO-1 (A), NQO1 (B), Srnx1 (C), and Gclc (D) genes. C: Control. *$p < 0.02$, **$p < 0.005$, ***$p < 0.001$.

4.1.19. Protein Expression Analysis

Since the gene expression analysis demonstrated the significant positive effects of M6, and M6 10 kDa on antioxidant gene expressions, the study proceed with the evaluation of these compounds' effects on the protein expression of Nrf-2. Activation of Nrf-2 concludes with the translocation of Nrf-2 from the cytoplasm to the nucleus. So that, after the treatment of products to cells for 4 h, nuclear, and cytoplasmic proteins were isolated from cells by using NE-PER™ Nuclear and Cytoplasmic Extraction Kit. Furthermore, proteins were separated based on their sizes via SDS-PAGE and, blotted on a nitrocellulose membrane by the Western Blot method. Membrane footages were analyzed after incubation with proper antibodies and the intensity of bands was determined, obtained data were normalized with β-actin data. Sulforaphane (SUL) was used as the positive control. M6 and M6 5-10 kDa both stimulated the nuclear accumulation of Nrf-2; protein expression of

nuclear Nrf-2 increased by 1.3, and 1.8 fold with the treatment of M6 250, and 500 µg/mL, respectively, in a dose-dependent manner. Also, 250, and 500 µg/mL doses of M6 5-10 kDa increased the Nrf-2 protein expression nearly by 1.3, and 1.4 fold, respectively (Fig. 20). Considering the fact that positive control SUL increased nuclear Nrf-2 expression approximately up to 1.2 fold, fermented *Spirulina* hydrolysate and its 5-10 kDa UF fraction may have potential indirect antioxidant activities.
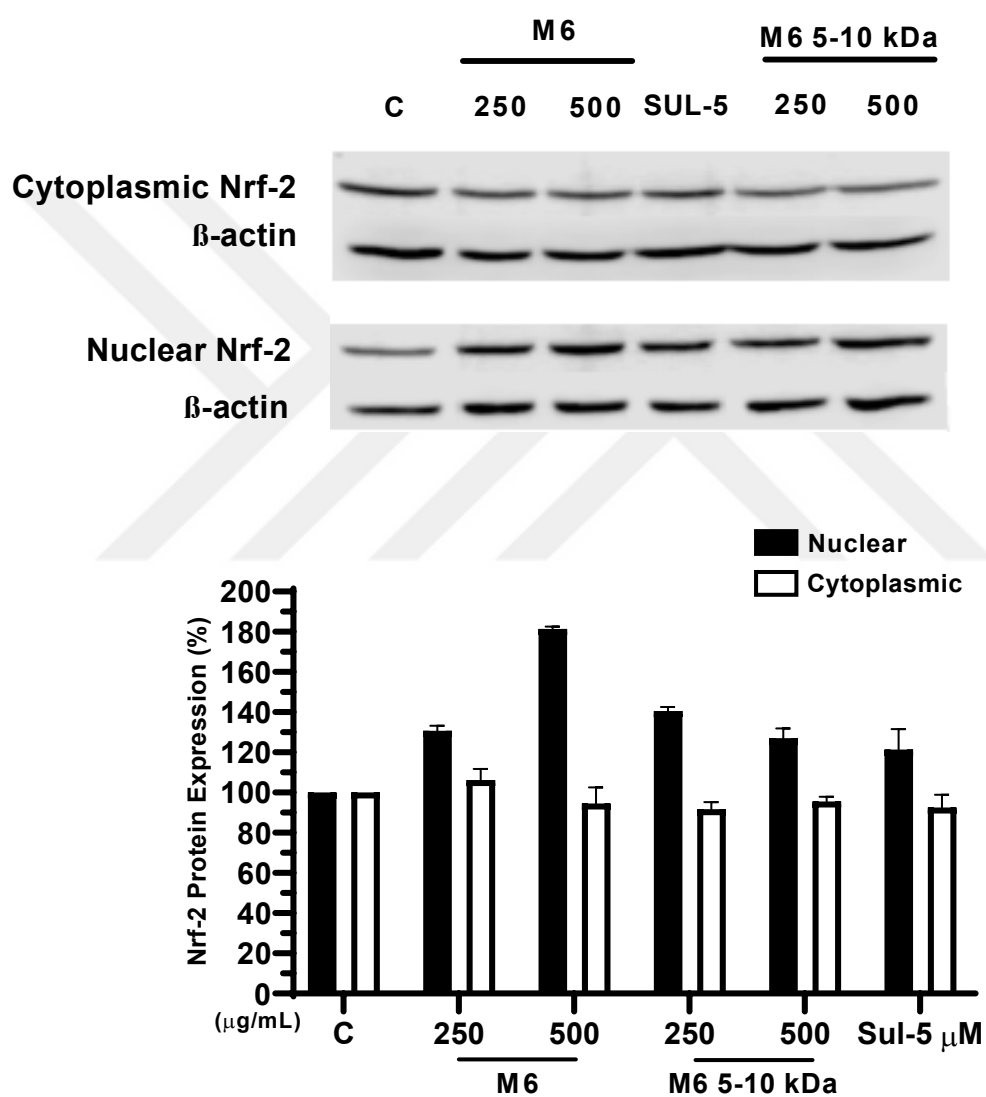

Figure 20. Effects of M6, and M6 5-10 kDa, on the protein expression of Nrf-2 at 4 h. C: Control, SUL: Positive control.

4.2. Discussion

This study was constructed with the aim of investigating the potential anti-inflammatory, anticancer, and indirect antioxidant effects of fermented *Spirulina* protein hydrolysate and bioactive peptide fractions as well as to elucidate the mechanism of actions at the molecular level of these products were demonstrated in a scientific basis. Since it is implicated that the novel supplements in process of development need to be investigated in terms of cytotoxicity and biocompatibility for the safety of further nutraceutical applications (Mohammadi et al., 2022), cytotoxic effects of all the fermentation products presented within this study were evaluated in healthy cell line HUVEC. Cytotoxicity data were representing a positive perspective on the biocompatibility of fermented *Spirulina* products, determined safe doses of optimization fermentation products, bioreactor fermentation products, UF fractions, and fermented *Spirulina* peptide fractions did not indicate cytotoxicity on healthy cells. Moreover, in this study, fermented and unfermented *Spirulina* were compared in terms of their bioactivities. With respect to data on cytotoxicity, mix-fermented hydrolysate demonstrated no significant cytotoxicity while the unfermented group decreased the cell viability nearly by 30% on healthy cells (Fig. 5C). This may be explained by the detractive effects against harmful molecules and toxins of product attained by the fermentation process (Garofalo et al., 2022). Correlatively, bioreactor fermentation hydrolysate M4 promoted the HUVEC cell viability nearly 1.8 fold where unfermented control product M3 did not imply a significant effect.

Furthermore, another advantageous effect of fermentation evaluated was enhanced, specific cytotoxicity of fermented products as compared to unfermented products on colon cancer cell line. For this matter, M6 inhibited the HT-29 cell viability with an IC_{50} value of 238.8 µg/mL while the calculated IC_{50} value of M5 was 1863 µg/mL i.e. fermentation process enhanced the cytotoxicity of *Spirulina* on colon cancer cells by 7.8 fold. M6 also showed cytotoxicity on prostate and breast cancer cells (Fig. 9), however, it was not as effective as it was on colon cancer cells. Additionally, one of the considerations in the scope of this study was to make a prediction on the activity of BAPs that may be produced by fermentation. So, bioactivity-oriented fractionation steps were followed to deduce if the activity was based on peptides. When M6, M6 5-10 kDa, and FR1 were compared, IC_{50} values (238.8, 248.3, and 250.4 µg/mL, respectively) of these products were relatively close; yet increased as the hydrolysate was further purified, this may be interpreted as potential

anticancer effects of fermented products were not originated from one-specific peptide but the synergistic effects of peptides and other byproducts of fermentation.

M6 and mix-fermented *Spirulina* products showed distinguished NO suppressive effects compared to the LPS-induced control groups. On the other hand, all unfermented products increased the released NO level up to nearly 230%, 177%, and 227% for KM-C, LH-C, and mix-C, respectively. Correlatively, it was shown that polysaccharides purified from *Spirulina platensis* induced NO production without the presence of LPS on RAW 264.7 cells (X. Wu et al., 2020). Considering the correlation, NO suppressive properties of mix product may be attributed to the acquisition of the fermentation process. Also, it was observed that the NO suppressive potential of products decreased as the product further fractionated. UF fractions of M6 and fermented *Spirulina* peptide fractions did not demonstrate a significant suppressive effect compared to the M6 or mix. This can be another substantiation showing the effect of synergistic effects of peptides and other byproducts of fermentation.

CHAPTER 5
CONCLUSION

In the scope of this thesis, the anticancer, anti-inflammatory, and indirect antioxidant effects of fermented *Spirulina platensis* hydrolysates and its peptide fractions were evaluated for their potential uses in both pharmaceutical and nutraceutical purposes. FS products produced by three different microbial fermentations (by *Lactobacillus helveticus*, *Kluyveromyces marxianus,* and in the combination of both microorganisms) were tested and FS product by mix was chosen according to its promising bioactive properties. With the chosen microbial group (mix), different batch operational bioreactor productions were performed in the bioreactor by our collaborative research group, and potential anti-cancer activities of bioreactor FS products (M2, M4, M6, M8) and their unfermented correspondings (M1, M3, M5, M7) were evaluated on HT-29 cell line. Since the M6 significantly and dose-dependently decreased the viability of colon cancer cells, the cytotoxic effects of M6 and its unfermented counterpart M5 were further evaluated on HT-29, MCF-7, PC-3, and HUVEC cell lines in a wider dose range. Among bioreactor fermentation products, M6, which demonstrated the most promising bioactive properties was used for UF fractionations (>2 kDa, 2-5 kDa, 5-10 kDa). Moreover, each UF fraction was tested and the selected fraction 5-10 kDa was further fractioned into its fractions by reverse phase high-performance liquid chromatography (RP-HPLC). In the last step, the effects of promising products (M6, M6 5-10 kDa, FR1, and FR2) on antioxidant genes were evaluated. Also, the effects of M6 and the 5-10 kDa UF fraction of M6 on the Nrf-2 activation were presented by using the Western Blot technique.

Spirulina hydrolysate fermented by the mixed culture of *Kluyveromyces marxianus* and *Lactobacillus helveticus*, and the bioreactor-level product called M6 presented a distinctive potency on LPS-induced NO production. Also, M6, UF fractions (5-10 kDa) of M6, and fractions obtained from UF fractions (5-10 kDa) of M6 as FR1 and FR2 by RP-HPLC demonstrated selective cytotoxicity on HT-29 cells. Yet, products did not affect the expression of Caspase-3 which suggest that the mechanism underlying the cell death may not be related to apoptosis. Therefore, future studies can proceed to evaluate the type of cell death. Furthermore, M6 and UF fractions of M6 belonging to the 5-10 kDa range

significantly enhanced the expressions of antioxidant and detoxification genes including Nrf-2, HO-1, NQO1, Srnx1, and Gclc, and also, M6 demonstrated a potential Nrf-2 activatory effect by stimulating the nuclear accumulation of Nrf-2. Overall, due to potential NO suppressing, anticancer effects, and high capacity to upregulate antioxidant and detoxification genes, fermented *Spirulina platensis* may be considered in further studies focusing on the development of nutraceuticals.

REFERENCES

Ansar, W., & Ghosh, S. (2016). *Biology of C reactive protein in health and disease. Biology of C Reactive Protein in Health and Disease*. Retrieved from https://doi.org/10.1007/978-81-322-2680-2

Balkwill, F., Charles, K. A., & Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell*, 7(3), 211–217. Retrieved from https://doi.org/10.1016/j.ccr.2005.02.013

Bent, R., Moll, L., Grabbe, S., & Bros, M. (2018). Interleukin-1 beta—A friend or foe in malignancies? *International Journal of Molecular Sciences*, 19(8). Retrieved from https://doi.org/10.3390/ijms19082155

Betoret, E., Betoret, N., Vidal, D., & Fito, P. (2011). Functional foods development: Trends and technologies. *Trends in Food Science and Technology*, 22(9), 498–508. Retrieved from https://doi.org/10.1016/j.tifs.2011.05.004

Chen, Q. M., & Maltagliati, A. J. (2018). Nrf2 at the heart of oxidative stress and cardiac protection. *Physiological Genomics*, 50(2), 77–97. Retrieved from https://doi.org/10.1152/physiolgenomics.00041.2017

Czerwonka, A., Kaławaj, K., Sławińska-Brych, A., Lemieszek, M. K., Bartnik, M., Wojtanowski, K. K., … Rzeski, W. (2018). Anticancer effect of the water extract of a commercial Spirulina (Arthrospira platensis) product on the human lung cancer A549 cell line. *Biomedicine & Pharmacotherapy*, 106, 292–302. Retrieved from https://doi.org/10.1016/J.BIOPHA.2018.06.116

Dallagnol, A. M., Pescuma, M., De Valdez, G. F., & Rollán, G. (2012). Fermentation of quinoa and wheat slurries by Lactobacillus plantarum CRL 778: proteolytic activity. *Applied Microbiology and Biotechnology 2012 97:7*, 97(7), 3129–3140. Retrieved August 25, 2021 from https://doi.org/10.1007/S00253-012-4520-3

Dartsch, P. C. (2008). Antioxidant potential of selected Spirulina platensis preparations. *Phytotherapy Research*, 22(5), 627–633. Retrieved August 25, 2021 from https://doi.org/10.1002/PTR.2310

Dinarello, C. A., & van der Meer, J. W. M. (2013). Treating inflammation by blocking

interleukin-1 in humans. *Seminars in Immunology*, 25(6), 469–484. Retrieved from https://doi.org/10.1016/j.smim.2013.10.008

Dirsch, V. M., Stuppner, H., & Vollmar, A. M. (1998). The griess assay: Suitable for a bio-guided fractionation of anti- inflammatory plant extracts? *Planta Medica*, 64(5), 423–426. Retrieved from https://doi.org/10.1055/s-2006-957473

Domínguez Díaz, L., Fernández-Ruiz, V., & Cámara, M. (2020). The frontier between nutrition and pharma: The international regulatory framework of functional foods, food supplements and nutraceuticals. *Critical Reviews in Food Science and Nutrition*, 60(10), 1738–1746. Retrieved from https://doi.org/10.1080/10408398.2019.1592107

El-Baz, F. K., El-Senousy, W. M., El-Sayed, A. B., & Kamel, M. M. (2013). In vitro antiviral and antimicrobial activities of Spirulina platensis extract. *Journal of Applied Pharmaceutical Science*, 3(12), 52–56. Retrieved from https://doi.org/10.7324/JAPS.2013.31209

Garofalo, C., Norici, A., Mollo, L., Osimani, A., & Aquilanti, L. (2022). Fermentation of Microalgal Biomass for Innovative Food Production. *Microorganisms*, 10(10), 1–20. Retrieved from https://doi.org/10.3390/microorganisms10102069

He, L., He, T., Farrar, S., Ji, L., Liu, T., & Ma, X. (2017). Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cellular Physiology and Biochemistry*, 44(2), 532–553. Retrieved from https://doi.org/10.1159/000485089

Jakubczyk, A., Karaś, M., Baraniak, B., & Pietrzak, M. (2013). The impact of fermentation and in vitro digestion on formation angiotensin converting enzyme (ACE) inhibitory peptides from pea proteins. *Food Chemistry*, 141(4), 3774–3780. Retrieved from https://doi.org/10.1016/J.FOODCHEM.2013.06.095

Jamnik, P., Mahnič, N., Mrak, A., Pogačnik, L., Jeršek, B., Niccolai, A., … Ulrih, N. P. (2022). Fermented Biomass of Arthrospira platensis as a Potential Food Ingredient. *Antioxidants*, 11(2). Retrieved from https://doi.org/10.3390/antiox11020216

Jhan, J. K., Chang, W. F., Wang, P. M., Chou, S. T., & Chung, Y. C. (2015). Production of fermented red beans with multiple bioactivities using co-cultures of Bacillus subtilis and Lactobacillus delbrueckii subsp. bulgaricus. *LWT - Food Science and Technology*, 63(2), 1281–1287. Retrieved from https://doi.org/10.1016/J.LWT.2015.03.107

Kiziltan, H. S., Bayir, A. G., Taspinar, O., Yucesan, G., Tastekin, D., Sonmez, F. C., … Mayadagli, A. (2015). Radioprotectant and cytotoxic effects of spirulina in relapsed verrucous vulvar cancer: A case report. *Alternative Therapies in Health and Medicine*, 21(April 2017), 68–72.

Koníčková, R., Vaňková, K., Vaníková, J., Váňová, K., Muchová, L., Subhanová, I., … Vítek, L. (2014). Anti-cancer effects of blue-green alga Spirulina platensis, a natural source of bilirubin-like tetrapyrrolic compounds. *Annals of Hepatology*, 13(2), 273–283. Retrieved from https://doi.org/10.1016/s1665-2681(19)30891-9

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal*, 16(9), 945–960. Retrieved from https://doi.org/10.1016/j.idairyj.2005.10.012

Koyande, A. K., Chew, K. W., Rambabu, K., Tao, Y., Chu, D. T., & Show, P. L. (2019). Microalgae: A potential alternative to health supplementation for humans. *Food Science and Human Wellness*, 8(1), 16–24. Retrieved from https://doi.org/10.1016/j.fshw.2019.03.001

Lafarga, T., Sánchez-Zurano, A., Villaró, S., Morillas-España, A., & Acién, G. (2021a). Industrial production of spirulina as a protein source for bioactive peptide generation. *Trends in Food Science and Technology*, 116(July), 176–185. Retrieved from https://doi.org/10.1016/j.tifs.2021.07.018

Lafarga, T., Sánchez-Zurano, A., Villaró, S., Morillas-España, A., & Acién, G. (2021b). Industrial production of spirulina as a protein source for bioactive peptide generation. *Trends in Food Science & Technology*, 116, 176–185. Retrieved from https://doi.org/10.1016/J.TIFS.2021.07.018

Lee, H.-B., Kim, E.-K., Park, S.-J., Bang, S., Kim, T. G., & Chung, D. (2010). Isolation and Characterization of Nicotiflorin Obtained by Enzymatic Hydrolysis of Two Precursors in Tea Seed Extract. *Journal of Agricultural and Food Chemistry*, 58(8), 4808–4813. Retrieved August 25, 2021 from https://doi.org/10.1021/JF9045182

Madhusudan, S., Muthuramalingam, S. R., Braybrooke, J. P., Wilner, S., Kaur, K., Han, C., … Ganesan, T. S. (2005). Study of etanercept, a tumor necrosis factor-alpha inhibitor, in recurrent ovarian cancer. *Journal of Clinical Oncology*, 23(25), 5950–5959.

Retrieved from https://doi.org/10.1200/JCO.2005.04.127

Moayedi, A., Hashemi, M., & Safari, M. (n.d.). Valorization of tomato waste proteins through production of antioxidant and antibacterial hydrolysates by proteolytic Bacillus subtilis: optimization of fermentation conditions. Retrieved from https://doi.org/10.1007/s13197-015-1965-2

Mohammadi, M., Soltanzadeh, M., Ebrahimi, A. R., & Hamishehkar, H. (2022). Spirulina platensis protein hydrolysates: Techno-functional, nutritional and antioxidant properties. *Algal Research*, 65(March), 102739. Retrieved from https://doi.org/10.1016/j.algal.2022.102739

Otterbein, L. E., Foresti, R., & Motterlini, R. (2016). Heme Oxygenase-1 and Carbon Monoxide in the Heart: The Balancing Act between Danger Signaling and Pro-Survival. *Circulation Research*, 118(12), 1940–1959. Retrieved from https://doi.org/10.1161/CIRCRESAHA.116.306588

Panigrahy, D., Gilligan, M. M., Serhan, C. N., & Kashfi, K. (2021). Resolution of inflammation: An organizing principle in biology and medicine. *Pharmacology and Therapeutics*, 227. Retrieved from https://doi.org/10.1016/j.pharmthera.2021.107879

Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling*, 24(5), 981–990. Retrieved from https://doi.org/10.1016/j.cellsig.2012.01.008

Samarakoon, K., & Jeon, Y. J. (2012). Bio-functionalities of proteins derived from marine algae — A review. *Food Research International*, 48(2), 948–960. Retrieved from https://doi.org/10.1016/J.FOODRES.2012.03.013

Shah, N. P. (2007). Functional cultures and health benefits. *International Dairy Journal*, 17(11), 1262–1277. Retrieved from https://doi.org/10.1016/j.idairyj.2007.01.014

Smieszek, A., Giezek, E., Chrapiec, M., Murat, M., Mucha, A., Michalak, I., & Marycz, K. (2017). The influence of Spirulina platensis filtrates on caco-2 proliferative activity and expression of apoptosis-related microRNAs and mRNA. *Marine Drugs*, 15(3). Retrieved from https://doi.org/10.3390/md15030065

Sun, J., Zhang, X., Broderick, M., Fein, H., Instruments, W. P., & International, S. (2003). JEI SON 2003sensors-03-00276. *Sensors*, 3, 276–284. Retrieved from

http://www.mdpi.net/sensors

Tripathi, P., Tripathi, P., Kashyap, L., & Singh, V. (2007). The role of nitric oxide in inflammatory reactions. *FEMS Immunology and Medical Microbiology*, 51(3), 443–452. Retrieved from https://doi.org/10.1111/j.1574-695X.2007.00329.x

Vaňková, K., Marková, I., Jašprová, J., Dvořák, A., Subhanová, I., Zelenka, J., … Vítek, L. (2018). Chlorophyll-mediated changes in the redox status of pancreatic cancer cells are associated with its anticancer effects. *Oxidative Medicine and Cellular Longevity*, 2018(Figure 1). Retrieved from https://doi.org/10.1155/2018/4069167

Vieira, C. P., Rosario, A. I. L. S., Lelis, C. A., Rekowsky, B. S. S., Carvalho, A. P. A., Rosário, D. K. A., … Conte-Junior, C. A. (2021). Bioactive Compounds from Kefir and Their Potential Benefits on Health: A Systematic Review and Meta-Analysis. *Oxidative Medicine and Cellular Longevity*, 2021. Retrieved from https://doi.org/10.1155/2021/9081738

Wallace, J. S. (2000). Increasing agricultural water use efficiency to meet future food production. *Agriculture, Ecosystems and Environment*, 82(1–3), 105–119. Retrieved from https://doi.org/10.1016/S0167-8809(00)00220-6

Wang, Q., Liu, F., Chen, X., Yang, Z., & Cao, Y. (2020). Effects of the polysaccharide SPS-3-1 purified from Spirulina on barrier integrity and proliferation of Caco-2 cells. *International Journal of Biological Macromolecules*, 163, 279–287. Retrieved from https://doi.org/10.1016/j.ijbiomac.2020.06.203

Wang, Z., & Zhang, X. (2016a). Characterization and antitumor activity of protein hydrolysates from Arthrospira platensis (Spirulina platensis) using two-step hydrolysis. *Journal of Applied Phycology*, 28(6), 3379–3385. Retrieved from https://doi.org/10.1007/s10811-016-0881-9

Wang, Z., & Zhang, X. (2016b). Inhibitory effects of small molecular peptides from Spirulina (Arthrospira) platensis on cancer cell growth. *Food and Function*, 7(2), 781–788. Retrieved from https://doi.org/10.1039/c5fo01186h

Wang, Z., & Zhang, X. (2017). Isolation and identification of anti-proliferative peptides from Spirulina platensis using three-step hydrolysis. *Journal of the Science of Food and Agriculture*, 97(3), 918–922. Retrieved from https://doi.org/10.1002/jsfa.7815

Wolkers, H., Barbosa, M., Kleinegris, D., Bosma, R., & Wijffels, R. H. (2011). Microalgae: the green gold of the future. *Green Raw Materials*, (October 2016), 9–31.

Wong, C. C., Baum, J., Silvestro, A., Beste, M. T., Bharani-Dharan, B., Xu, S., … Svensson, E. C. (2020). Inhibition of IL1β by canakinumab may be effective against diverse molecular subtypes of lung cancer: An exploratory analysis of the CANTOS trial AC. *Cancer Research*, 80(24), 5597–5605. Retrieved from https://doi.org/10.1158/0008-5472.CAN-19-3176

Wu, Q., Liu, L., Miron, A., Klímová, B., Wan, D., & Kuča, K. (2016). The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: an overview. *Archives of Toxicology*, 90(8), 1817–1840. Retrieved from https://doi.org/10.1007/s00204-016-1744-5

Wu, X., Liu, Z., Liu, Y., Yang, Y., Shi, F., Cheong, K. L., & Teng, B. (2020). Immunostimulatory Effects of Polysaccharides from Spirulina platensis In Vivo and Vitro and Their Activation Mechanism on RAW246.7 Macrophages. *Marine Drugs*, 18(11). Retrieved from https://doi.org/10.3390/md18110538

Yang, S., & Lian, G. (2020). ROS and diseases: role in metabolism and energy supply. *Molecular and Cellular Biochemistry*, 467(1–2), 1–12. Retrieved from https://doi.org/10.1007/s11010-019-03667-9
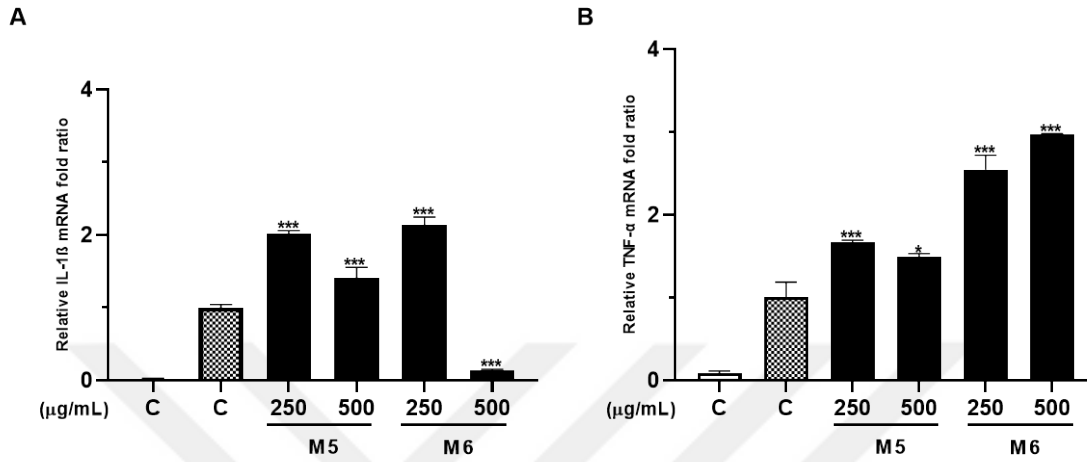
Figure 1. Effects of fermented (M6) and unfermented (M5) *Spirulina platensis* on IL-1β (A), and TNF-α (B) gene expressions of LPS-induced RAW 264.7 cells. C: Control, C: Control (Only LPS). *p < 0.02, **p < 0.005, ***p < 0.001.
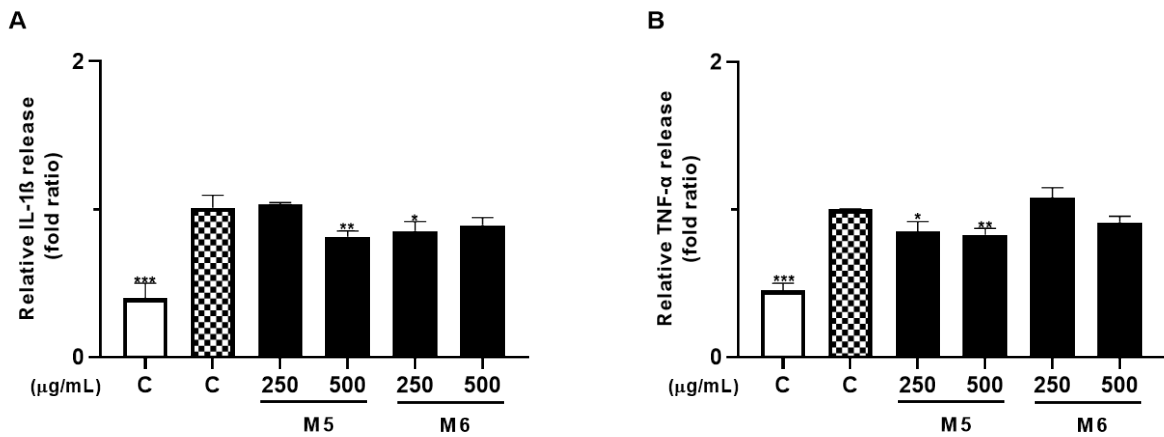

Figure 2. Effects of fermented (M6) and unfermented (M5) *Spirulina platensis* on IL-1β (A), and TNF-α (B) release of LPS-induced RAW 264.7 cells. C: Control, C: Control (Only LPS). *p < 0.02, **p < 0.005, ***p < 0.001.