PHYSIOLOGIC EFFECTS OF THE GOLDEN THISTLE (*Scolymus hispanicus* L.) HYDROMETHANOLIC EXTRACTS: OUTCOMES OF PHYTOCHEMICAL HEALTH BENEFITS

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ABSTRACT

PHYSIOLOGIC EFFECTS OF THE GOLDEN THISTLE (*Scolymus hispanicus* L.) HYDROMETHANOLIC EXTRACTS: OUTCOMES OF PHYTOCHEMICAL HEALTH BENEFITS

This dissertation aimed to screen the beneficial health effects of a hydromethanolic extract (GTE) obtained from the golden thistle (*S. hispanicus* L.) on different health conditions, including type 2 diabetes, inflammation, cancer, and wound healing. First, 1 mg/mL GTE resulted in 6.94% chlorogenic acid (CGA) bioavailability with $(1.82\pm0.07)x10^{-6}$ cm/s apparent permeability on differentiated CaCo-2 cells. Then, 1 mg/mL GTE prompted 39.4-42.6% less glucose efflux and 49-66% less GLUT2 mRNA expressions on CaCo-2 cells. In the systemic inflammation model, pre-treatments of 50-500 μg/mL GTE reduced some inflammatory markers after 0.5 μg/mL lipopolysaccharide (LPS) inflammation induction for 12 h on RAW 264.7 cells. Reductions in 30-53%, 32-45% and 16-36% ranges for nitric oxide, tumor necrosis factor-α, and interleukin-6 (IL-6) were determined, respectively. Additionally, same GTE concentrations were pre-treated with the CaCo-2 cells in the colonic inflammation model. 15.5-19.5% and 8.7-17.3% less IL-6 and IL-8 cytokine releases were detected from CaCo-2 cells, respectively. The wound healing model of 3T3-L1 mouse fibroblasts revealed that 40-80 μg/mL root bark extract resulted in enhanced wound closures with significant differences in the cell cycle distributions. As the most significant result, G2 phase distributions were 1.8% and 12.5% in the negative and positive control samples, respectively. The root bark extract treatments of 10, 40, and 80 μg/mL resulted in 6.6%, 7.1%, and 9.1% in increasing concentrations. Finally, 4 mg/mL GTE application to CaCo-2 human adenocarcinoma cells caused 78.4% reduced cell viability, a cell cycle arrest, and increased early and late apoptotic properties. Overall results suggest that *S. hispanicus* L. has functional molecules that influence cellular regulations and have potential beneficial health effects.

ÖZET

ŞEVKETİBOSTAN (*Scolymus hispanicus* L.) HİDROMETANOLİK EKSTRAKTLARININ FİZYOLOJİK ETKİLERİ: FİTOKİMYASALLARIN SAĞLIK FAYDALARI ÜZERİNE BULGULAR

Bu tez çalışması, şevketibostandan elde edilmiş hidrometanolik özütlerinin (ŞBÖ) sağlık üzerine etkilerinin tip 2 diyabet, inflamasyon, kanser ve yara iyileşmesi gibi modellerde taranmasını amaçlamaktadır. İlk olarak, farklılaştırılmış CaCo-2 hücreleri üzerine 1 mg/mL ŞBÖ uygulaması, %6.94 klorojenik asit biyoyararlılığı ve $(1.82\pm0.07)x10^{-6}$ cm/s geçiş değerlerinin bulunmasını sağlamıştır. Ardından, 1 mg/mL ŞBÖ, aynı hücre hattının kullanımıyla oluşturulan antidiyabetik aktivite modelinde, glukoz geçiş değerlerini %39.4-42.6 aralığında, GLUT2 mRNA ifadelerini ise %49-66 aralığında düşürmüştür. Sistemik inflamasyon modelinde, 50-500 µg/mL konsantrasyon aralığında ŞBÖ ön-uygulaması, daha sonra 12 saat boyunca 0.5 µg/mL lipopolisakkarit (LPS) verilerek inflamasyonun indüklendiği RAW 264.7 hücrelerinde bazı inflamasyon belirteçlerinin düşüşünü sağlamıştır. Nitrik oksit (NO), tümör nekroz faktörü alfa (TNF-α) ve interlökin 6 (IL-6) salınımlarında sırasıyla %30-53, %32-45 ve %16-36 aralıklarında düşüşler saptanmıştır. Ek olarak, kolonik inflamasyon modelinde aynı konsantrasyonlar CaCo-2 hücrelerine ön-işlem olarak verilmiş ve sırasıyla IL-6 ve IL-8 sitokin salınımlarında %15.5-19.5 ve %8.7-17.3 aralıklarında düşüşler tespit edilmiştir. Yara iyileşmesi modelinde, 40-80 µg/mL şevketibostan kök kabuğu ekstraktının 3T3-L1 hücrelerine uygulanmasının, yara kapanması ve hücre döngüsü üzerinde etkili olduğu bulunmuştur. G2 fazında negatif ve pozitif kontroller sırasıyla %1.8 ve %12.5 iken, 10, 40 ve 80 µg/mL ŞBÖ uygulamalarından sırasıyla %6.6, %7.1 ve %9.1 dağılımları elde edilmiştir. Son olarak, 4 mg/mL ŞBÖ'nün CaCo-2 insan kolon kanser hattı hücrelerinde hücre canlılığını %78.4 oranında düşürerek hücre döngüsünde tutuklanmanın yanı sıra, erken ve geç apoptotik aktivitelerin de artışına neden olduğu gözlenmiştir. Sonuç olarak, *S. hispanicus* L., hücresel faaliyetleri etkileyebilen ve sağlık üzerine yararlı etkilere sahip olma potansiyeli bulunan fonksiyonel moleküller barındırmaktadır.

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ABBREVIATIONS

AAE	Ascorbic Acid Equivalents
ABC	ATP-Binding Casette
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACF	Aberrant Crypt Focci
Ae	Aerial
A549	Human Pulmonary Carcinoma Cell Line
A431	Human Epidermoid Carcinoma Cell Line
AMPK	5’ Adenosine Monophosphate-Activated Protein Kinase
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Collaboration
AP-1	Activator Protein 1
APC	Adenomatous Polyposis Coli
ATCC®	Americal Type Culture Collection
AUC	Area Under Curve
BSA	Bovine Serum Albumin
BSAE	Bovine Serum Albumin Equivalents
CaCo-2	Human Adenocarcinoma Cell Line
CAM	Chorioallantoric Membrane
CBG	Cytosolic Beta Glycosidase
CE	Catechin Equivalents
cDNA	Complementary DNA
CGA	Chlorogenic Acid
CVD	Cardiovascular Diseases
C32	Human Melanoma Cell Line
DAD	Diode Array Detector
DEPC	Diethyl Pyrocarbonate
DM	Diabetes Mellitus
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid

DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ETAE	Aegean Agricultural Research Institute, Ege Tarımsal Araştırma Enstitüsü
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalents
GDM	Gestational Diabetes Mellitus
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
GLUT	Glucose Transporter
GRAS	Generall Regarded as Safe
G1	First Growth Phase of the Cell Cycle
G2	Second Growth Phase of the Cell Cycle
GTE	Golden Thistle Extract
HaCaT	Human Keratinocyte Line
HBSS	Hank's Balanced Salt Solution
HCT15 and HCT16	Human Colorectal Cell Line
HepG2	Human Liver Hepatocellular Carcinoma Cell Line
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
HT-29	Human Colorectal Adenocarcinoma Cell Line
HTC 16	Human Colorectal Carcinoma Cell Line
IBD	Inflammatory Bowel Diseases
IC_{50}	Inhibition Concentration of 50%
IDF	International Diabetes Federation
IL	Interleukin
IM	Inflammatory Medium
INS-1β	Rat Insulinoma-Derived Cell Line
K562	Human Leukemia Lymphoblast Cell Line

KG1a	Human Erythroleukemia Cell Line
KRAS	Kirsten Rat Sarcoma Virus
L5178Y	Mouse Lymphoma Cell Line
LPH	Lactase-Phlorizin Hydrolase
LPS	Lipopolysaccharide
M	Mitosis
MAPK	Mitogen Activated Protein Kinase
MCF-7	Human Adenocarcinoma Cell Line from Breast Tissue
MCT	Monocarboxylic Acid Transporter
MD	Medical Doctor
ME	Mannose Equivalents
MEM	Minimum Essential Medium
MetS	Metabolic Syndrome
MPF	Maturation-Promoting Factor
MODY	Maturing-Onset Diabetes of the Young
MRC-5	Human Embryonal Lung Fibroblast Cell Line
mRNA	Messenger Ribonucleinc Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N.A.	Not Applied
NCI-H460	Human Large Lung Cancer Cell Line
NCI-N87	Human Gastric Carcinoma Cell Line
NED	N-(1-naphthyl)ethylenediamine
NFκB	Nuclear Factor Kappa B
NK cells	Natural Killer Cells
NO	Nitric Oxide
P_{app}	Apparent Permeability
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC3	Human Prostatic Carcinoma Cell Line
PGE	Prostaglandin
PHA	Phytohaemmagglutinin
PI	Propidium Iodide
PIK3CA	Phosphatidylinositol 3-Kinase

PPARγ	Proliferator-Activated Receptor Gamma
PTP1B	Protein Tyrosinase Phosphatase B
RAW 264.7	Murine Macrophage Cell Line
RB	Root Bark
RE	Rutin Equivalents
RI	Refractive Index
RI	Root Internal
RKO	Human Colon Carcinoma Cell Line
RPMI 1640	Roswell Park Memorial Institute
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
RUBIA	Circum-Mediterranean Ethnobotanical and Ethnographic Heritage in Traditional Technologies, Tools, and Uses of Wild and Neglected Cultivated Plants for Food, Medicine, Textiles, Dyeing, and Handicrafts
S	DNA Replication Phase in the Cell Cycle
SEM	Standard Error Mean
SGLT1	Sodium Dependent Glucose Transporter 1
STAT3	Signal Transducer and Anc Activator of Transcription 3
SYBR-Green™	DNA Binding Dye in RT-qPCR
TAGEM	Turkish General Directorate of Agricultural Research and Policies, Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü
TE	Trolox Equivalents
TEAC	Trolox Equivalents Antioxidant Capacity
TEER	Transepithelial Electrical Resistance
TGFβ	Transforming Growth Factor Beta
TIG-119	Human Fibroblast Cell Line
TLC	Thin Layer Chromatography
TNF-α	Tumor Necrosis Factor Alpha
TP53	Tumor Protein 53
Triton X-100	2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TÜİK	Turkish Statistical Institute, Türkiye İstatistik Kurumu

3T3-L1	Mouse Fibroblast Cell Line
UNESCO	United Nations Educational, Scientific and Cultural Organization
VEGF	Vascular Epidermal Growth Factor
VOLD	Veno-occlusive Liver Disease
WI-38	Diploid Human Cell Line of Fibroblasts
Wnt	Wingless-Related Integration Site

CHAPTER 1

INTRODUCTION

At the present time, the researchers have concentrated on discovering and designing novel formulations that help health and well-being due to the increased demand of consumers. For that purpose, various molecular groups of plant and animal origin have been investigated. The active molecules or molecular groups present in these resources have been known as functional molecules. Functional molecules cover a wide range of effects on health and well-being, including the prevention of diseases such as cancer, cardiovascular diseases, and Alzheimer's disease (Kim, Lee, and Lee 2010, Nishino et al. 2005, Visioli, Borsani, and Galli 2000, Zhang et al. 2015). Among the functional molecules, plant-based molecular groups draw significant attention due to easier production properties, availability, and variety. There is a wide market share of plant-based molecules in the economy. Indeed, 25% of the drugs and supplements that have been marketed are from natural plant sources (Süntar, 2020). It has been reported that up to 80% of the molecular groups that have been utilized only in cancer treatments have been obtained from plants (Dehelean et al., 2021).

Another approach in this research area is to investigate the commonly known diets. One of the important diets in this context is the Mediterranean diet (Corrêa et al. 2020, Degner, Papoutsis, and Romagnolo 2009). Although the main effect of the Mediterranean diet has been associated with the raw consumption of olive oil, it is also known that the diet is very rich in various plants. Mediterranean plants have been utilized for both culinary and medical purposes. The introduction of wild edible plants in the Mediterranean diet has been started with famine and war times, however, the tradition has continued so far. In 2013, the Mediterranean Diet was inscribed as an Intangible Cultural Heritage of Humanity by the United Nations Educational, Scientific and Cultural Organization (UNESCO) (Motti et al. 2020).

One of the most appreciated wild edible plants in the Mediterranean diet is the golden thistle (*Scolymus hispanicus* L.). It has been consumed in countries like Turkey, Greece, Spain and other Mediterranean countries with lamb or eggs, and sometimes, consumed directly in salads (Polo et al. 2009). Apart from gastronomic area, golden thistle plant has

an important role in the local medicine of the area. Golden thistle ethanolic extract was the first licensed plant-based drug in the years between 1934 and 1978. It was formulated by MD Cemil Şener, named as Lityazol Cemil, and used for removing calculi from kidneys. However, due to the lack of the wild edible plant and loss of Dr Şener, the production has come to an end. Today, the factory that Lityazol Cemil had been produced was turned into a museum in Manisa, Turkey.

Diseases including obesity, type 2 diabetes mellitus (T2DM), cancer, different kinds of allergies, and other immune disorders have been increased with the changed lifestyle. A sedentary lifestyle has been adopted in many countries as a result of urbanization and technology. In addition to the sedentary lifestyle, other factors like smoking and genetic heritage also increase the risk of developing diseases. The term "metabolic syndrome (MetS)" was proposed covering cardiovascular diseases (CVD), T2DM, and obesity. The authorities believe that there might be a pro-inflammatory state before developing these conditions (Eckel, Grundy, and Zimmet 2005). In general, diseases under MetS occur together in susceptible individuals (Samson and Garber 2014).

In the prevention of MetS, lifestyle change is recommended with increased physical activity and a healthy diet (Strasser 2013). Healthy diet targets not only losing weight, but also intake of several micronutrients that helps the health and well-being status. Micronutrients include vitamins and minerals as well as phytochemicals. Various phytochemical groups like phenolic substances, certain vitamins, carotenoids, terpenoids, antioxidant minerals like selenium or zinc can be listed. Among them, vitamin C and vitamin E has been proved to be highly antioxidant both *in vitro* and *in vivo* conditions (Burton et al. 1983, Chen et al. 2000, Huang et al. 2002). On the other hand, *in vivo* antioxidant data on other phytochemicals such as phenolic substances are controversial due to their poor bioavailability (Gao and Hu 2010). Yet, these compounds possess high antioxidant activity in test tube methods and thus, the research on developing more bioavailable antioxidants is ongoing (Ajazuddin and Saraf 2010, Amalraj et al. 2021, Zhong and Shahidi 2011).

MetS is associated with various types of conditions. However, the most prevalence is seen in T2DM. Associated with insulin resistance and elevated blood glucose levels, T2DM affects many individuals worldwide. International Diabetes Federation (IDF) publishes a periodic and updated atlas about the prevalence of the disease (IDF 2022). Due to its link with MetS, the most important recommendations in the prevention and treatment of

T2DM is the change in the lifestyle with less glucose consumption and more physical activity. On the other hand, medicinal approaches might be needed in some of the individuals. The drugs such as metformin and acarbose can be prescribed (Wang et al. 2013). In addition, recent studies suggest that the phytochemical compounds may help the individuals in the prevention of elevated blood glucose levels (Ahangarpour, Sayahi, and Sayahi 2019). These compounds may act as digestive enzyme inhibitors and/or inhibitors of glucose absorption in molecular levels (Teoh and Das 2018).

Any molecule that is consumed by a person should become dissolved in human body fluids and liberated for absorption. The term *bioaccessibility* refers to the portion of a food constituent that is digested and liberated in the gastrointestinal tract. On the other hand, *bioavailability* implies the portion that the enterocytes absorb and transfer to the bloodstream. *Bioactivity* is the last step of this sequence and stands for the portion that reaches the target tissue in the human body (Dima et al. 2020, Parada and Aguilera 2007). Consequently, consumption of a molecule does not necessarily mean that all the molecules should reach the target tissue. The evaluation of the bioaccessible, bioavailable and bioactive portions of a food matrix, therefore, becomes important in this context. Researchers can determine the bioaccessible portion with the help of *in vitro* digestion methods conducted in test tube. The next step is determining the bioavailable portion and requires cell culture studies. The cultures of mammalian enterocyte-like cells can be utilized for this purpose. However, determining the bioactivity depends on the *in vivo* studies including clinical trials or animal models.

The somatic cells follow the same cell cycle route for division. Basically, they grow, replicate their deoxyribonucleic acid (DNA), and divide (Alberts 2017). However, there are various other factors that affect this route. Some of these factors may suppress the division process, and some others may accelerate it. In either way, external factors such as dietary components play a role. In diseases like cancer, the suppression of the cell cycle of the cancerous cells might help the individual during the treatment. On the other hand, for situations like wounding, it is helpful if the cell cycle and division process accelerates. During the cell cycle, there are four main phases that the cell goes through. And while the cell passes these cell cycle phases; the natural checkpoint mechanisms intercede and determine if that cell would go further division or not. Phytochemical components might affect these cell cycle checkpoints. They might cause a cell cycle arrest and prevent the cell from mitosis in relatively high doses, or they might act on *vice versa* on lower doses.

The ability of phytochemicals on proliferation and wound healing mechanism provides researchers for utilizing them for wound healing purposes (Sivamani et al. 2012, Thangapazham, Sharad, and Maheshwari 2016, Shah and Amini-Nik 2017). The phytochemical compounds that generally have proliferative activity, might have some anti-inflammatory effects, as wounds always generates inflammation in the certain area (Shah and Amini-Nik 2017).

Human body have two main immune systems: innate, and adaptive immune systems (Dempsey, Vaidya, and Cheng 2003). These two systems work together when the host is infected or wounded, resulting in healing of the affected parts. There are some immune cells that constitute these two immune systems. The immune cells communicate with each other by releasing specific protein molecules called cytokines. All these reviewed actions are called as *inflammation*. Inflammation is a very important mechanism that every individual should have to eliminate the foreign microbial attack to the body. On the other hand, the immune system sometimes goes out of control and starts releasing excessive cytokines even there is no microbial invasion or wounds. This excessive immune system activity results in different kinds of allergies and other severe conditions (Tucci et al. 2010). In conclusion, inflammation is a situation that should be kept under control.

Cancer has a huge impact on world population. During the cancer treatment, medical approaches like chemotherapy, radiotherapy and/or combination of these two approaches have been very successful for most of the cases. On the other hand, these treatments are very destructive and cause a significant decrease in the life quality of the patients due to agony. In addition to the chemotherapy and radiotherapy, in non-invasive cancer types, surgery is also utilized. When all these treatment methods are considered together, it becomes very clear that the treatment of cancer condition requires huge amounts of patience and finance. Therefore, research on the prevention and alternative treatments on cancer therapies have become important. The approach of preventing the disease before cancer transition have been associated with a healthy lifestyle that is rich in fruits and vegetables, as well as consumption of the extract supplements of these resources (Akhtar et al. 2020). There is no doubt that the prevention approach is more cost-effective than the cancer therapies that are applied after the condition. It also helps the consumers generating a healthy lifestyle and better quality of life. The plant-based molecules are widely used in raw consumption and supplement production for that purpose.

According to the literature information given above, it can be said that the MetS related diseases as well as cancer and inflammation, are highly interconnected with each other and inflammation as the starting point. Any molecular group extracted from a plant source might have effects on different disease models. Therefore, newly researched molecular groups may be utilized in scanning studies for the determined disease models. In fact, consumers expect a better health and well-being condition after taking the supplements and formulations. However, research and regulations on that topic is generally concerning for those different molecular groups in our country. In addition, various powders or formulations for these health claims are marketed in local shops and bazaars without any scientific research on the area. Furthermore, most of these raw materials are marketed in open containers, creating a food safety issue. It is crucial to investigate the health effects of a plant-based molecule with scientific research methods, rather than individual experiences. The molecule should be carefully tested, the scientific evidence should be established, and lastly, safe formulations with proper doses should be designed for the end product before supplying it to the end user.

Therefore, in this thesis project, it was aimed to screen the possible health effects of a crude methanolic extract. Golden thistle plant was chosen as the raw material and hydromethanolic extracts was obtained from the parts of the plant. In the first step, the macronutrient and phytochemical compositions of the raw material were determined. Then, antioxidant activity of the golden thistle hydromethanolic extract has been investigated by test tube methods. Moreover, effects on various disease models and conditions have been investigated utilizing *in vitro* cell culture techniques. Among them, first an *in vitro* bioavailability model has been designed and the bioavailable fraction of the possible functional groups were tested. Secondly, the effects of the extract on an *in vitro* antidiabetic model were determined. The systemic and colonic inflammation models were investigated on two cell culture models of inflammation. The research was followed by determining the effects of the extracts on proliferation and wound healing models. Moderate doses were utilized up to this point. For the investigation of anticarcinogenic model, relatively higher doses of the extracts were used on colon cancer cells assuming that the gastrointestinal tract was suitable for such high doses independent from the bioavailable portion.

CHAPTER 2

THE FAMILY OF ASTERACEAE, THE GENUS *Scolymus*, AND *S. hispanicus* L.

Due to the increased number of non-communicable diseases in the last few decades, the concept of a healthy lifestyle has become more important for many consumers. The possibility of mass production and globalization with developing technology led to the embrace of the Western diet worldwide. However, the latest evidence showed that the lack of minimally processed foods and a sedentary lifestyle resulted in increased amounts of metabolic diseases, including obesity, type 2 diabetes mellitus (T2DM), cancer, neurodegenerative and cardiovascular diseases, as well as other related conditions. Therefore, the nutritionists recommended a cutback to minimally processed foods with more physical activity. From this evidence, there is an emerging interest in traditional diets and lifestyles among consumers. One of the most famous traditional dietary habits recommended and applied worldwide is the Mediterranean lifestyle.

2.1. The Mediterranean Diet

In 1960, Angel Keys observed that the populations in countries like Greece and Italy suffered less from non-communicable diseases and linked this observation with their dietary habits (Giugliano and Esposito 2008). The concept of the Mediterranean Diet arose from that observation. Today, it has been accepted as a healthy and sustainable dietary habit (Burlingame and Dernini 2011). The Mediterranean diet has been considered one of the ideal dietary profiles for preventing diseases as it meets the essential criteria for a healthy diet (Sofi et al. 2013, Trichopoulou and Vasilopoulou 2000). Although the dietary habits can differ in different Mediterranean countries (e.g., the consumption of pasta is higher in Italy whereas the consumption of seafood is higher in Spain), the most important common points of the diet are the abundance of virgin olive oil, high intake of fruits, vegetables, legumes, nuts, and minimally processed foods, moderate intake of fish and shellfish, alcohol and fermented dairy products, and lower intakes of meat and

processed meat products (Martínez-González et al. 2017, Trichopoulou and Vasilopoulou 2000, Willett 2006). Sofi et al. (2013) reviewed the relationships between the Mediterranean diet and non-communicable diseases. In this study, the evidence on the prevention of cardiovascular diseases is stated, whereas the preventive effect on neurodegenerative diseases like Alzheimer's and Parkinson's diseases has been reported. Furthermore, the Mediterranean diet has been considered a nonpharmaceutical option for the treatment of Diabetes Mellitus (DM). On the other hand, both Sofi et al. (2013) and Martínez-González et al. (2017) stated that there should be more evidence for the cancer-diet relationship. A similar approach was remarked in another study reporting 25%, 15%, and 10% prevention of colorectal, breast, and prostate cancers with the help of the Mediterranean diet, indicating the need for more studies (La Vecchia 2004).

It has been suggested that the preventive effect of the Mediterranean diet might be primarily linked to the consumption of a high amount of minimally processed foods and raw virgin olive oil. These two foods are mainly obtained without much processing, and most of the antioxidant compounds like tocopherols from olive oil and phenolic acids are highly preserved before consumption. Another advantage of minimally processed vegetables is the intake of relatively high amounts of dietary fiber.

The Mediterranean diet is primarily linked to the dietary habits of the Cretan people of Greece. Throughout history, the island of Crete has been one of the world's most important exporters of olive oil (Adıyeke and Adıyeke 2021). The massive production of minimally processed olive oil and the biodiversity of agricultural products resulted in lower mortality rates among the Cretan people. However, a very important part of the Mediterranean countries is often underrated. The wild edible plants constitute a significant portion of the Mediterranean lifestyle. Although the beginning point of the consumption of wild edible plants is unclear, these self-growing plants are highly consumed during wars and famine times in history. Most wild edible foods are associated with Crete, yet the consumption is sometimes more frequent in the other Mediterranean countries. A wide range of wild edible plants from various plant families are consumed not only for gastronomic reasons but also as a traditional phytomedicine. For example, plants from Lamiaceae, Asteraceae, Liliaceae, Apiaceae, Fabaceae, Poaceae, Rosaceae, and Myrtaceae are known and consumed by local people (González-Tejero et al. 2008). In these families, Asteraceae is a well-known family with some of its plants originating and/or endemic to the Mediterranean region.

2.2. Asteraceae Family

The family of Asteraceae provides many species in the Mediterranean diet as both agricultural and wild-edible aspects. Asteraceae is an extensive family in the plant kingdom with approximately 1000 genera and over 25 000 species (Bessada, Barreira, and Oliveira 2015). The members of this family have been not only used in the traditional folkloric medicine in especially Mediterranean, Eastern Europe, and Asia Minor (Anatolia) regions, some countries like Germany, Czech Republic, France, and Switzerland included some of the Asteraceae plants in their pharmacopeias (Achika et al. 2014, Bessada, Barreira, and Oliveira 2015). In an ethnobotanical field survey of the medicinal plants used by the local populations in Mediterranean countries (the project RUBIA, Circum-Mediterranean Ethnobotanical and Ethnographic Heritage in Traditional Technologies, Tools, and Uses of Wild and Neglected Cultivated Plants for Food, Medicine, Textiles, Dyeing, and Handicrafts), 21.6% of all plants interviewed belonged to the Asteraceae family (Hadjichambis et al. 2008). Achika et al. (2014) reviewed some of the activities of the family and concluded that the members of Asteraceae had wound healing, antioxidant, antidiabetic, cytotoxic, antiulcer, immunosuppressive, and anti-inflammatory, as well as antispasmodic activities both *in vitro* and *in vivo*. The most widely studied and reported members of Asteraceae may be listed as follows: yarrow (*Achillea millefolium* L.), wormwood (*Artemisia absinthium* L.), echinacea (*Echinacea purpurea*), dandelion (*Taraxacum officinale*), billy goat weed (*Ageratum conyzoides* L.), tridax daisy (*Tridax procumbens* L.), black-jack (*Bidens pilosa* L.), and pot marigold (*Calendula officinalis* L.) (Bessada, Barreira, and Oliveira 2015, Morales et al. 2012, Saini, Chauhan, and Kaushik 2020). In addition, the common daisy (*Bellis perennis* L.) and chicory (*Cichorium intybus*) are two additional examples from Asteraceae that are widely studied. There are also plants that are Native to the Mediterranean, Anatolian, and European regions, such as groundsel (*Senecio vulgaris* L.) as well as sowthistle (*Sonchus oleraceus* L.) with traditionally known and/or scientifically studied properties on health and well-being. The properties that some of these listed Asteraceae plants possess are summarized briefly.

Yarrow (*Achillea millefolium* L.) is probably the best-known Asteraceae plant worldwide. The plant is named as *civanperçemi* in Turkish and is used in folkloric medicine not only in the Mediterranean but also in other areas of Turkey. There are 23 different usages in

the traditional medicine of yarrow only in Kırklareli province (Kültür 2007). In medicinal plant knowledge studies, yarrow has been reported to be used against inflammation, wounds, headaches and pain, and hepatobiliary disorders (Düsman et al. 2013). In addition, alcoholic extracts or pure compounds isolated from the yarrow have been investigated *in vivo*. According to these data, yarrow was effective against the ulcerative lesions in gastric mucosa, has immunosuppressive activity (Saeidnia et al. 2011), and possesses antidiabetic activity in streptozotocin-induced diabetic rats (Rezaei et al. 2020). Moreover, both Düsman et al. (2013) and Cavalcanti et al. (2006) reported no cytotoxicity of the yarrow exposures in animal studies. Akram (2013) summarized the traditional uses, chemical constituents, and pharmacological research of yarrow in their work.

Another well-known Asteraceae plant is wormwood (*Artemisia absinthium* L., *pelinotu* in Turkish). It has been used in traditional medicine for various purposes such as antispasmodic, anthelmintic, antifungal, antidepressant activities, etc. (Batiha et al. 2020, Bora and Sharma 2011). Bhat et al. (2019) reported that the plant has been used in Greek, Unani, and Ayurveda medicines. One of the traditional uses of wormwood is to induce abortions in unwanted pregnancies (Nigam et al. 2019). On the other hand, the usage of this plant is very speculative due to the presence of thujone. Because thujone causes neurotoxic effects in long term and central nervous system disorders in high dose exposures (Batiha et al. 2020). Although wormwood is used as a flavoring agent in a specific spirit, the beverage was banned in many countries because of the effects of thujone. Oral applications of wormwood, therefore, are not recommended (Bhat et al. 2019). On the contrary, scientific research and drug development studies might still benefit from the wormwood. The hydromethanolic extract of the plant was reported to be cytotoxic to cancerous cell lines like breast (MCF-7) and colon (HTC16) cancer cells *in vitro* (Gordanian et al. 2014, Sultan et al. 2020, Sura et al. 2011). In the study of Sultan et al. (2020), wormwood was also shown to have wound healing activity as well as a regulatory effect on inflammation markers. Furthermore, crude aqueous extract of wormwood showed inhibitory activity on a worm in mammalians which is *Hymenolepis nana*, both *in vitro* and *in vivo* (Beshay 2018). The essential oil formulation of wormwood in nanochelates suppressed Leishmaniasis (Tamargo et al. 2017) and showed hepatoprotective activity by regulating antioxidant systems and immunological markers (Amat, Upur, and Blažeković 2010). These studies confirm that the wormwood of Asteraceae can be utilized in drug development studies, as it has functional potential. On

the other hand, the utilization of the wormwood by the local populations is speculative due to thujone presence.

Marigold plants are very popular in terms of landscaping in the Mediterranean and other European countries. Pot marigold (*Calendula officinalis* L.) could probably be one of the most studied marigold plants in most research areas. There are various effects of the pot marigold to be suggested by both *in vitro* and *in vivo* studies. Furthermore, mostly cosmetic formulations containing pot marigold have been marketed. The plant is known as *aynısefa* in Turkish and is sold in many local herbalist stores. The best-known activity of the pot marigold is its wound healing effect. However, various other effects have been documented. Khalid and Teixeira da Silva (2012) reviewed the health effects of pot marigold, including antiseptic, anti-inflammatory, antiedematous, anticancer, and many others, in detail. The wound healing activity of the pot marigold has been studied in rats, mice, and *in vitro* models. There is a review about pot marigold only dedicated to wound healing activity (Givol et al. 2019). One of the proposed mechanisms of the wound healing activity is via nuclear factor kappa B (NFκB) and increased interleukin 8 (IL-8) secretion when the human immortalized keratinocytes were treated with the ethanolic extract of the plant (Nicolaus et al. 2017). In another *in vitro* study, pot marigold inhibited collagen degradation and human matrix metalloproteinase-2 enzyme (collagenase) activity of human gingival fibroblasts (Saini et al. 2012). Gunasekaran, Nayagam, and Natarajan (2020) applied pot marigold ointment in a rat excision model and obtained increased wound contraction, collagen synthesis, and better inflammatory parameters. Furthermore, Dinda et al. (2016) investigated the effects of pot marigold hydroethanolic extract both *in vitro* and *in vivo* with suggestions of possible active molecules. Kharat et al. (2021) also prepared pot marigold containing chitosan/polyethylene oxide scaffolds for rat wounds and reported increased collagen synthesis as well as other improved wound healing parameters. Ointment of pot marigold was compared with an *Aloe vera* cream against diaper dermatitis in a clinical study, and the researchers reported that the application of pot marigold ointment caused less rash than that of aloe vera cream with no adverse effects (Panahi et al. 2012). The plant was also utilized for irritant contact dermatitis in adults in a clinical study and was reported to have a protective effect (Fuchs et al. 2005). In literature, cream formulations for better wound healing purposes have been prepared (Bernatoniene et al. 2011, Mahyari et al. 2016). In addition to the wound healing activity, the anti-diabetic potential was also interesting. Ebrahimi et al. (2019)

gave pot marigold hydroalcoholic extract to streptozotocin-induced diabetic rats. The researchers have found a direct effect on beta cell recovery and increased insulin levels with better liver markers. Furthermore, pot marigold resulted in lower blood glucose levels, urine sugar, and serum lipids in alloxan-induced diabetic rats (Chakraborthy, Arora, and Majee 2011). In a study, the saponins of pot marigold helped reduce the blood glucose levels and inhibited gastric emptying in oral glucose-loaded mice, as well as provided an inhibition in mucosal lesions obtained by indomethacin (Yoshikawa et al. 2001). This study shows the relationship between anti-diabetic and anti-inflammatory activities. In another study investigating the anti-inflammatory potential of the pot marigold, both rat and Chorioallantoic Membrane (CAM) model were used, and stimulation of fibroplasia and angiogenesis were reported (Parente et al. 2012). Tanideh et al. (2016) showed that the administration of pot marigold resulted in a reduction in inflammatory conditions of the rats having acetic acid-induced ulcerative colitis. Additionally, pot marigold extracts and fractions have been tested on leukemic cells, and cytotoxic activities were reported (Wegiera et al. 2012). Other cancerous cell lines were investigated in the study of Jiménez-Medina et al. (2006) with possible mechanisms of action. The researchers reported induction in the cell cycle, apoptosis *in vitro*, and tumor suppression *in vivo*. Cruceriu, Balacescu, and Rakosy (2018), (Frankič, Salobir, and Salobir 2009) recommended the possible usage of pot marigold for the side effects of radiotherapy in palliative care in their work and highlighted the need for further studies before applications. Similarly, the antioxidant potential of the plant was studied in a pig model with polyunsaturated fatty acid, given lipid peroxidation induced DNA damage (Frankič, Salobir, and Salobir 2009). In that study, it was observed that the propylene glycol extracts of the petals were better and comparable with the results of vitamin E. Alnuqaydan et al. (2015) studied the hydrogen peroxide oxidative damage on human skin cells (HaCaT) and reported both dose and time-dependent protection. In an antioxidative *in vivo* study of mice liver, it was observed that catalase and intracellular glutathione levels were high after 30 days of treatment (Preethi, Kuttan, and Kuttan 2006). One of the most interesting effects of pot marigold could be the anti-HIV activity as the plant provided protection against HIV-1 *in vitro* and reduced the HIV-1 reverse transcriptase enzyme in a cell-free environment (Kalvatchev, Walder, and Garzaro 1997). Lagarto et al. (2011) reported low acute and subchronic toxicity of pot marigold in rats. The usage of pot marigold formulations was accepted in generally regarded as safe (GRAS) status (Verma et al. 2018). The European Medicinal Agency recognized pot marigold as an

herbal medicinal product (Cruceriu, Balacescu, and Rakosy 2018). Indeed, pot marigold has been used in local and global drug formulations for a long time (Arora, Rani, and Sharma 2013).

In most European and Middle East regions, including the Mediterranean area, the common daisy (*Bellis perennis* L.) is used as an herbal tea. The plant is known as adi papatya in Turkish and is used for wound healing, rheumatism, eczema, eye diseases and tonsillitis (Karakas et al. 2017), as well as hemorrhoids (Tuzlacı and Erol 1999), sore throat, and common cold (Kizilarslan Hancer and Ozhatay 2012). However, the best-known effect of the common daisy is the sedative effect in traditional medicine. Karakaş research group has a wide spectrum of studies about the common daisy *in vitro* or *in vivo*. For instance, they verified the wound healing effect of the plant *in vivo* with a model on Wistar rats with n-butanol fraction (Karakaş et al. 2012), the antiproliferative activity of methanolic extract on MCF-7 human breast cancer cell line (Karakaş et al. 2015) and reported cellular antioxidant activity (Karakas et al. 2017). In addition, Marques, Melo, and Freitas (2012) suggested that the ethanolic extract of the common daisy possesses antidepressant activity in mice depression model, and Zangeneh et al. (2018) reported that the common daisy reduced hematotoxicity and nephrotoxicity in CCl_4-induced mice model. Furthermore, common daisy induced GLUT4 translocation and lower blood glucose levels in two different models. The researchers of the previously mentioned study confirmed one of the possible mechanisms of the antidiabetic effect (Haselgrübler et al. 2018). For the anti-obesity effect, the methanolic extract and saponin fractions suppressed serum triglyceride elevations when the mice were fed with olive oil (Morikawa et al. 2008). The same research group also reported a promoted collagen synthesis in common daisy-treated normal human dermal fibroblasts for the methanolic extract (Morales et al. 2014). Lastly, the incorporation of the common daisy into sun protection agents has been suggested after complete research due to its ability to protect keratinocytes from ultraviolet damage (Souza de Carvalho et al. 2021).

One of the most appreciated plants from Asteraceae is the chicory (*Cichorium intybus*). There is a lot of research investigating the health effects of chicory both *in vitro* and *in vivo*. It is native to the Mediterranean area and used as a coffee additive with inulin as the primary compound (Choudhary, Kaurav, and Chaudhary 2021). In Turkish, the chicory is known as *hindiba*. The plant was used in the food industry and has GRAS (Generally Regarded as Safe) status (Perović et al. 2021). Either leaf or the root parts of the chicory

have been used for culinary, medicinal, and cosmetic purposes so far (Puhlmann and de Vos 2020). In local medicine, the plant was used for fever, hepatic failure, diarrhea, cough, cancer, hangover, liver enlargement and disorders, jaundice, gallstones, mild chronic skin diseases, other digestive disorders, gout, and rheumatism (Choudhary, Kaurav, and Chaudhary 2021, Mulabagal et al. 2009). Wound healing activity is the most well-known activity of chicory in Turkish local medicine, which has been scientifically investigated in rat excision and incision models (Süntar et al. 2012). The wound healing activity was also studied in a rat study of gastric ulcer model, and stomach protection was the obtained result (Gürbüz et al. 2002). In addition, usage as animal feed and for diabetes and cirrhosis have been stated (Street, Sidana, and Prinsloo 2013). It was reported as antioxidant, anti-inflammatory, anticancer, antiprotozoal, antidiabetic, antimicrobial, immunological and cardiovascular protective, hyperlipidemic, gastroprotective, analgesic, anthelmintic, productive enhancer, and with wound healing activity, as well as a laxative, detoxifying, invigorating, blood-cleansing effects (Janda et al. 2021, Nwafor, Shale, and Achilonu 2017). The methanolic chicory extract was investigated on acetaminophen-induced liver damage in rats and reported as hepatoprotective (Gilani, Janbaz, and Javed 1993). Likewise, in most of the reported research, chicory ameliorated carbon tetrachloride-induced liver damage in a rat study (Elgengaihi et al. 2016, Fallah Huseini et al. 2011). The hepatic injury was induced by nitrosamine precursors in another animal study, and the hepatoprotective effect was reported again with beneficial effects in antioxidant mechanisms (Hassan and Yousef 2010). Another widely studied effect of the chicory is its antidiabetic potential. In a clinical study, the consumption of chicory root extract has been shown to be potentially effective against diabetes and improved bowel movements (Nishimura et al. 2015). In streptozotocin-induced diabetic rats, the chicory extract was effective in oral glucose test, serum glucose levels, and glucose-6-phosphatase activity, but the researchers reported that there was no effect on insulin levels (Pushparaj et al. 2007). Similar effects have been reported in the study of Azay-Milhau et al. (2013). In that research, an insulin release was observed in the *in vitro* part of the study, which used rat insulinoma-derived INS-1β cells, while no insulin release was observed in rats *in vivo*. In a similar study, the methanolic chicory extract was studied on high fat diet and streptozotocin induced diabetic rats and 3T3-L1 fibroblasts (Muthusamy et al. 2010). It was shown that the extract altered insulin sensitivity and plasma metabolic profile. In fibroblasts, the extract inhibited adipogenesis and altered the glucose uptake. The mechanism of the process was suggested as the inhibition of protein tyrosinase

phosphatase 1B (PTP1B). In a cell culture study with CaCo-2 human adenocarcinoma cell line, a dose-dependent antioxidant activity was reported (D'Evoli et al. 2013). On the contrary, the transepithelial electrical resistance (TEER) values decreased with increasing concentrations for the same cell line under oxidative stress (Azzini et al. 2016). The chicory extract was investigated for kidney damage and improvements in kidney structure and functions have been reported (Elmasry et al. 2020). The anti-inflammatory potential of the Asteraceae family members and most other plant sources has been studied in literature because of inflammatory state being the first step in most diseases. In that context, the chicory was studied in a rat paw edema model, and it was shown to reduce the inflammatory cytokines like TNF-α, IL-6, and IL-1, as well as an increase in the antioxidant enzymes like catalase and glutathione peroxidase (Rizvi et al. 2014). It was also shown that the ethanolic extract of chicory inhibited T cell stimulating activity of the dendritic cells at high concentrations yet modulated the cytokine release of the T cells at low concentrations (Karimi et al. 2014). On the other hand, the cytotoxic activities reported for chicory extracts are variable. The aqueous extract was incorporated into silver nanoparticles and showed cytotoxic activity on MCF-7 human breast cancer cell line (Behboodi et al. 2019). In contrast, chicory showed lower toxicity on T47D breast, RKO colon, and PC-3 prostate cancer cell lines (Nawab et al. 2011). In literature, a comprehensive review of the cytotoxic activity of the chicory and its extracts have been published by Imam et al. (2019), however, the reviewers suggested further studies for better understandings of the mechanism of action. Due to its inulin content, production of the short chain fatty acids at the end of colonic fermentation was also studied. It was reported that for the pigs consuming chicory on their diet, the abundant short chain fatty acids were lactic acid and butyrate for ileum and colon, respectively (Liu et al. 2012).

Groundsel (*Senecio vulgaris* L.) is a wild plant native to European and Asian areas yet grow in most of other regions. The plant was used for toothaches, stomachache, strangury, and jaundice (Mitich 1995) in folk medicine, but then restricted due to its toxic components (Xiong et al. 2014). The known Turkish name for groundsel is *kanarya otu*. Groundsel contains alkaloids, which are converted into hepatotoxic pyrrolizidine alkaloids in liver and the poisoning is cumulative (Mitich 1995). The consumption of long-term groundsel formulations causes veno-occlusive liver disease (VOLD) (Ortiz Cansado et al. 1995). Due to the toxicity of alkaloid substances, various fractions or extracts have been investigated on cancerous cell lines. Hammoda et al. (2016) reported

that the alkaloid containing chloroform fraction was effective on HCT-16 colorectal cancer cell line. Likewise, methanolic fraction was effective on CaCo-2 adenocarcinoma, n-hexane fraction was effective on C32 amelanotic melanoma and HepG2 hepatocarcinoma, and lastly dichloromethane fraction was effective on CaCo-2, HepG2, and COR-L23 non-small lung cancer cell lines, while no toxicity was detected on non-cancerous MRC-5 human fetal lung cell line (Conforti et al. 2006).

Sowthistle (*Sonchus oleraceus* L.) is an invasive weed that is native to Mediterranean area. The plant is known as *eşek marulu* in Turkish. There are various *in vitro* and *in vivo* studies investigating different health effects of the sowthistle extracts. For instance, it was investigated on an ulcerative colitis model *in vivo* and reported to possess antiulcerogenic activity (Alothman et al. 2018). The sowthistle extract was investigated for its antidiabetic as well as anti-inflammatory activity in many studies. For instance, Chen et al. (2019) studied streptozotocin induced diabetic model and tested some of the liver markers. The researchers found that the antioxidant system components superoxide dismutase and glutathione levels were increased, while inflammatory markers of TNF-α and IL-1β were decreased. Same research group also conducted a follow-up study suggesting possible molecular markers and pathways involved in the antidiabetic effect (Chen, Lin, Fan, Qian, et al. 2020), and developed a self-emulsifying delivery system with the sowthistle extract to overcome the poor bioavailability of the plant phenolics and tested the anti-inflammatory and antidiabetic effects on mice (Chen, Lin, Fan, Lv, et al. 2020, Chen, Lin, Xu, et al. 2020). The anti-inflammatory activity of the sowthistle was also reported by other researchers. In fact, Vilela, Bitencourt, et al. (2010) reported better results in anti-inflammatory activity than that of positive controls indomethacin and dexamethasone in a rat model. Additionally, the antinociceptive and antidepressant activities of the sowthistle extracts were also evaluated *in vivo* (Vilela, de Mesquita Padilha, et al. 2010, Vilela et al. 2009). The other studies for the sowthistle plant include antiobesity effect by stimulating the 5' adenosine monophosphate-activated protein kinase (AMPK) pathway (Chen et al. 2021), improved wound healing properties in a rat model (Prichoa, Roman, and Manfredini 2011), and nephroprotective effect against ischemia-reperfusion induced kidney injury in rats (Torres-González et al. 2018). The cytotoxic activity of various extracts and fractions of the sowthistle was studied too. Some of these might be summarized as reported cytotoxicities in L5178Y mouse lymphoma, PC33 rat brain cancer cells (Elkhayat 2009), NCI-N87 stomach cancer cells (Yin, Kwon, and Wang

2007), and in HepG2 hepatocarcinoma and K562 erythroleukemic cells through cell cycle arrest in G0/G1 checkpoint (Huyan et al. 2016). The extracts were also studied for its antioxidant activities as cellular antioxidant activity procedure with HepG2 (McDowell et al. 2011) as well as hydrogen peroxide induced oxidation in WI-38 human fibroblasts (Ou, Rades, and McDowell 2015). Chen, Teng, and Cao (2019) reported improved insulin sensitivity of HepG2 cells when treated with sowthistle and highlighted the presence of chlorogenic acid in their material. Yang et al. (2018) investigated the anti-inflammatory activity of the sowthistle and reported dose dependent reductions in inflammation markers like nitric oxide, prostaglandin (PGE2) and pro-inflammatory cytokines on an LPS-induced systemic inflammation model by using RAW 264.7 murine macrophages.

As can be seen from the examples, Asteraceae is a relatively wide family including many species from various genera. The genus *Scolymus* also belongs to the Asteraceae family and native to the coastal Mediterranean. There are three members of this family: golden thistle (*S. hispanicus* L.), spotted golden thistle (*S. maculatus* L.), and large flowered golden thistle (*S. grandiflorus*). Therefore, it is possible that the golden thistle plants might have one or few of these activities. The three species of the genus *Scolymus* have been explained with their main studied properties.

2.1.1. Golden Thistle (*Scolymus hispanicus* L.)

The most famous species of the genus *Scolymus* is the golden thistle plant (*S. hispanicus* L.). It is considered as a wild edible plant consumed in many Mediterranean, Macaronesian and Near Orient countries (Vázquez 2000). Golden thistle is known and consumed in Turkey (Ertuğ 2004), Greece (Pardo-De-Santayana, Tardío, and Morales 2005), Spain (Polo et al. 2009), Portugal (Barão and Dias 2010), Italy (Disciglio et al. 2017, Lentini and Venza 2007), Cyprus (Della, Paraskeva-Hadjichambi, and Hadjichambis 2006) and Morocco (Powell et al. 2014). Guarrera and Savo (2016) described the golden thistle as a “pan-Mediterranean food plant”.

From the culinary perspective, golden thistle is cooked as lightly fried with garlic, cured ham and/or scrambled eggs, or can be stewed with chickpea, potatoes and meat in Spanish cuisine (Polo et al. 2009). Another form of the consumption is the combination of the golden thistle root bark with asparagus and eggs (Melián et al. 2017). It is also used as a

saffron substitute or condiment in the same region (Tardío, Pascual, and Morales 2005). On the other hand, edible parts of the plant can be used in traditional pasta dishes in Italy (Nebel, Pieroni, and Heinrich 2006).

In Turkey, golden thistle plant is mainly known as *şevketibostan* and the root bark is used in the traditional meals of Mediterranean coastal cities with lamb meat. In the south region of Antalya, golden thistle is utilized as a component of traditional börek, a dish of stuffed pastry (Aksoy, Çelik, and Tunay 2019). Synonyms of the golden thistle plant in Turkish are *altın dikeni*, *akkız*, and *sarıcakız* (Kirimer et al. 1997). The plant is sold with the name *kenker* in Bodrum and Milas local bazaars (Ertuğ 2004). In other regions, sometimes the plant parts are used in pickles, fried dishes with eggs, or boiled before adding into salads (Kök et al. 2020). On the other hand, the most important usage of the golden thistle plant in our country was its utilization as a medicinal drug. The ethanolic extract of the golden thistle plant was licensed and named as Lityazol Cemil between the years of 1934 and 1978 (Tunalier, Kirimer, and Başer 2006). The formula was obtained by soaking the edible parts of the plant in ethanol by the inventor, Cemil Şener, MD. However, as the plant was wild, the shortages occurred in the production. As a result, the production was stopped after Dr Şener passed away. Today, the factory in which Lityazol Cemil was produced is a museum in Manisa, Turkey.

The plant golden thistle can also be used for traditional medicinal purposes. The infusion of the root part was reported to be effective against ulcer with diuretic properties in the local medicine (Ugurlu and Secmen 2008). In addition, it was used for liver and intestinal diseases, considered as an antidiabetic agent, and utilized against Malta fever and eye-infections (Berdja et al. 2021).

In addition to the usage in traditional medicine, there are some *in vitro* and *in vivo* studies investigating the effects of golden thistle or the extracts of the plant. The ethanolic extract exhibited an inhibitory effect on two mammalian hydrolases, α-amylase and α-glucosidase (Marmouzi et al. 2017). The inhibitory activity on these enzymes is generally regarded as the *in vitro* antidiabetic activity in literature. In the study of Kirimer et al. (1997), the plant constituents were tested on rat ileum preparations and the presence of taraxasteryl acetate were emphasized with antispasmodic and spasmogenic activities. In an *in vivo* study investigating the antidiabetic effects, the golden thistle given rats with hyperfatty diet reduced their body weights (Berdja et al. 2021). In addition, the researchers reported improved glucose tolerance and regulated hepatic inflammation

markers. The authors concluded that the golden thistle exerted hypoglycemic and hypolipidemic effects. Similarly, significantly lower fasting blood glucose levels were detected in streptozotocin-induced diabetic rats (Ozkol et al. 2013).

Figure 2.1. Golden Thistle (*Scolymus hispanicus* L.) After Harvest. The photographs were taken after harvesting the golden thistle plants cultivated in Menemen, İzmir by Aegean Agricultural Research Institute. (a) The whole plant, (b) An illustration showing the three parts of aerial, root bark, and root internal, and (c) Some leafy aerial parts and root bark as the plant parts used in traditional cuisine.

The chloroform fractions of golden thistle flowers were investigated for their anti-inflammatory activities on peripheral blood mononuclear cells (PBMC) stimulated with phytohaemmagglutinin (PHA) (Kandil et al. 2020). In that study, reductions in interleukin 6 (IL-6), IL-1β, and tumor necrosis factor alpha (TNF-α) was found among cytokines. In addition, it was reported that there was a dose-dependent decrease in NFκB p65 content when the cells were treated with various concentrations of golden thistle flower extracts.

Asteraceae members exert antimicrobial effects in various microorganisms. Therefore, the antimicrobial effects of the golden thistle plant were also studied by Aboukhalaf et al. (2020). The researchers reported that the plant was effective on *Enterococcus faecalis*, *Escherichia coli*, and *Cryptococcus neoformans*. However, no effect was reported against *Staphylococcus aureus*, *Pseudomonas* spp., and *Candida albicans* in the same study. In another investigation on antimicrobial activity, Petropoulos et al. (2019) reported higher bacteriostatic and bactericidal activity on *Bacillus cereus* and *Salmonella typhimurium* than the positive control of that study in 0.10-0.300 mg/mL concentration range.

Main compositional and macronutrient analyses on the golden thistle plant have been reported in literature. Among them, Marmouzi et al. (2017) revealed one of the most comprehensive studies investigating the macro- and micronutrients of the golden thistle

plant extracts. Another detailed analysis on the plant composition was conducted by García-Herrera et al. (2014) with a caloric value of approximately 167 kJ/100 g plant. In their study, Morales et al. (2014) investigated some Mediterranean medicinal plants including the golden thistle. By eliminating the inedible parts, the researchers reported main macronutrient compositions, organic acid profiles, tocopherol contents, and phytochemical composition. Furthermore, the total phenolic content of golden thistle extract was studied before and after an *in vitro* digestion procedure (Gonçalves et al. 2019). This study concluded that there were no significant changes after digestion in terms of total phenolic content. This finding is an indication that the mammalian hydrolases have no effect on glycosidic phenolic-sugar bonds present in the plant composition.

The complete phytochemical contents of the golden thistle plant are still unclear. Presence of p-coumaric, procatechuic, isochlorogenic, chlorogenic, and quercetin-3-O-(2’’-O-caffeoyl)-β-D-glucurono-pyranoside have been reported earlier (Sanz et al. 1993). Similarly, chlorogenic and caffeic acids were reported in golden thistle extracts in another study (Marmouzi et al. 2017). Rubio et al. (1995) reported isorhamnetin-3-O-α-L-rhamnopyranosyl-β-glucopyranoside and kaempferol-3-O-α-L-rhamnopyranosyl-β-D-glucopyranoside. Bakour et al. (2020) suggested that golden thistle might contain alkaloids in the structure, yet this suggestion still needs to be confirmed with further chemical detection, as the authors indicated.

Apart from the phenolic compounds, carotenoid and tocopherol contents of the golden thistle has been studied and lutein, β-carotene, as well as α- and γ-tocopherols have been reported (Vardavas et al. 2006a). The fatty acid composition was investigated by Vardavas et al. (2006b) and 33.7% saturated, 54.8% monounsaturated, 65.8% polyunsaturated fatty acid profile was reported with 1.06 omega-6 to omega-3 acid ratio in 100 g fresh plant. Another detailed fatty acid analysis was conducted by Morales et al. (2014). In addition, Sánchez-Mata et al. (2012) studied the organic acid composition of golden thistle and reported high abundance of oxalic acid with others like malic, citric, fumaric, ascorbic, dehydroascorbic acids. The volatile composition was studies by Servi (2019) and heneicosane, hexahydrofarnesyl acetone and phytol have been reported as the main volatile composition. Lastly, the main inorganic composition was studied and reported by Disciglio et al. (2017). In some of the studies from literature, taraxasterol and taraxasteryl acetate are the reported terpenoids in the composition of golden thistle plant.

Yet, the contents varied in a low range. According to Sarı et al. (2011), the taraxasteryl acetate content can be detected in higher amounts when the plant was flowered and the aerial part above the soil is dried. A food application of the golden thistle flour has been studied on wheat flour and extended rheological properties have been reported after incorporation (Dülger Altıner and Şahan 2021). This is the first study in literature incorporating the golden thistle in a food matrix.

In some of the Mediterranean countries, agricultural studies on cultivating the golden thistle plant were initiated. In Turkey, Turkish General Directorate of Agricultural Research and Policies (Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü, TAGEM) has been cultivated the plant in Menemen region (İzmir province) by Aegean Agricultural Research Institute (Ege Tarımsal Araştırma Enstitüsü, ETAE). Between the years of 2016 and 2017, some of the physiologic as well as agricultural properties of the crops have been investigated. Sari, Odabas, and Tutar (2012) conducted a study with the mathematical modeling of the golden thistle cultivation and Güllüdağ and Yoldaş (2015) studied the possible harmful pests during the cultivation. An agricultural cultivation research was also conducted in Hellenic Mediterranean University in Crete, Greece (Papadimitriou et al. 2019). The scientists of this study reported a new environmental-friendly technique of cultivation of golden thistle plant. In Greece, the genetic diversity of the golden thistle was also investigated (Psaroudaki et al. 2015). As the plant is an important element of the traditional cuisine in Spain, it was also cultivated in that country (Sari and Tutar 2010).

The golden thistle has also been experimented as a natural alternative sorbent in the prevention of environmental contamination. According to the studies of Barka et al. (2010), the dried powder of the whole plant was reported as an excellent biosorbent in absorbing Cadmium (II). The same researchers also studied the removal of textile dyes methylene blue and eriochrome black T from wastewater (Barka, Abdennouri, and Makhfouk 2011). Likewise, they reported that golden thistle powder was able to absorb these two dyes and can be used in wastewater treatment as a natural source.

2.1.2. Spotted Golden Thistle (*Scolymus maculatus*)

Spotted golden thistle (*Scolymus maculatus*) is one of the members of the genus Scolymus (Vázquez 2000). It differs from the golden thistle with some phenotypic properties in general, yet these two plants can still be considered as similar (Bermejo, León, and Programme 1994). There is limited information about the plant except for its growth area and phenotypic properties. Lev-Yadun (2016) stated that the spotted golden thistle has a zebra-like leaf structure for camouflage, a property that the golden thistle plant does not have. Although the spotted golden thistle was described as a wild edible plant in Greece and Balkan region (Kostić et al. 2020), Sarı et al. (2011) indicated that the plant was not used as a vegetable due to its different root structure. Like the golden thistle, spotted golden thistle was also utilized for wound healing purposes in Middle East regions (Saad and Said 2011). The spotted golden thistle is mainly listed in the observatory articles describing the plant diversity in a particular area. The important point in these publications is that the spotted golden thistle plant is detected mainly in contaminated soil such as roadsides (Domínguez et al. 2017, Galán et al. 2004). In addition, Casciaro and Damato (2007) investigated the effects of temperature and light conditions on the germinating seeds of the plant.

Apart from the botanical and agricultural studies about the spotted golden thistle, the plant was experimented in the textile dye removal just like the golden thistle. According to Bereksi and Benaissa (2019), spotted golden thistle can be utilized in the removal of methylene blue for wastewater treatment purposes. In addition, it was investigated as a protective agent to increase the shelf life of Valencia oranges (Gao et al. 2021). In that study, the water dipping method with the spotted golden thistle improved the quality characteristics and enhanced the durability of the oranges. On the other hand, Meriem et al. (2014) reported that the compounds present in the spotted golden thistle were effective on κ-, αs- and β-caseins. Therefore, the plant can be suggested as an alternative for rennet enzymes in cheesemaking process.

Abu-Lafi et al. (2019) obtained different fractions and extracts form the spotted golden thistle and detected some of the phytochemical compounds present in the plant. Among them, stigmasterol, γ-sitosterol, lupeol, lupeol acetate, and α-amyrin were emphasized because of their medicinal properties. In addition, the radical scavenging activities of the extracts were 0.37 mg/mL at minimum. The researchers lastly investigated the effects of

the extracts on *Staphylococcus aureus*, *Salmonella typhimurium*, and *Candida albicans* and reported the effective fractions obtained from the plant.

2.1.3. Large-Flowered Golden Thistle (*Scolymus grandiflorus*)

Among the genus *Scolymus*, the least information has been published for *S. grandiflorus*. In some sources, it was named as large-flowered golden thistle due to its larger flower size than that of *S. hispanicus*. The large-flowered golden thistle, *S. grandiflorus* was mentioned along with *S. hispanicus* L. in Vázquez (2000), and some other flora observation studies. It was reported to be grown wildly in Algeria, Italy, and Turkey and mentioned as a repellant to the livestock due to its spiny structure in North Sahara region in literature (Ahmed, Nasrallah, and Okkacha 2020, Bocchieri and Iiriti 2004, Ghezlaoui, Chemouri, and Benabadji 2016, Kaya and Başaran 2006, Le Houérou 2001, Sarı et al. 2011). From the culinary perspective, Guarrera and Savo (2016) and Lentini (2000) included the large-flowered golden thistle in their work. The plant has been reported to be used in omelets, bread, goat cheese or boiled and seasoned with salt and oil. On the other hand, in Bartın province, Turkey, the large-flowered golden thistle was reported to be endangered because of its harvesting by the local people due to its medicinal properties (Kaya and Başaran 2006). Although *S. hispanicus* and *S. maculatus* have been listed in the plants that have been cultivated (Kays 2011), *S. grandiflorus* was not reported among them.

Apart from the observational and ethnobotanical studies, Tomb (1975) investigated the morphologic structure of the plant seeds by using scanning electron microscopy. Furthermore, the essential oil composition of extract fractions from large-flowered golden thistle and *in silico* effects on vascular endothelial growth factor (VEGF) has been reported recently (Semaoui et al. 2020). In this study, the researchers suggest that the high amount of davanone content might have an affinity for binding to VEGF, which is an important factor in tumor angiogenesis. Therefore, the further *in vitro* and *in vivo* studies are needed to confirm these computational chemistry results, as the authors emphasized.

2.2. Concluding Remarks and Overall Aim of the Thesis

Not only the trend for a healthier lifestyle, but also for cultural concerns, wild edible plants are gaining attention in terms of their phytochemical constituents and health effects. There are various plant families that are utilized as wild edible plants and for local medicine in different regions of the world. The consumption of wild edible plants is an important part of the Mediterranean diet and lifestyle as well. And the golden thistle plant (*S. hispanicus* L.) is one of the most widely consumed for that manner in the overall diet. Belonging to the family of Asteraceae, the golden thistle plant might have a potential in health and well-being conditions. As some important Asteraceae plants possess cytotoxic, anti-inflammatory, antimicrobial, and antidiabetic activity either *in vitro* or *in vivo*, there is a possibility that the golden thistle plant may have some of these activities due to its phytochemical composition.

Therefore, an overall screening study was aimed in this thesis project. For that purpose, first a proximate study was conducted and the moisture, soluble protein and carbohydrate, crude fiber, lipid, and ash content of the samples were determined. Then, individual extracts from the three parts of the golden thistle plant were obtained, and phytochemical composition in terms of total phenolic content, as well as flavonoid and tannin contents were tested. Third, antiradical capacities of the extracts were investigated against ABTS and DPPH radicals. The ferric reducing antioxidant power was also contained in this part. The abundant phenolic compound was determined in the following part by using high pressure liquid chromatography (HPLC) and an *in vitro* bioavailability test was applied to test the bioavailable portion of the phenolic composition present in the extracts. Then, as the models of antidiabetic activity, both starch digestibility and glucose efflux model were applied to the human enterocyte cells to investigate the effects on glucose release as well as absorption when the golden thistle plant is incorporated. As the Asteraceae plants are well known for their anti-inflammatory activities, two inflammation models were selected as systemic and colonic inflammation models to be tested. Utilizing the murine macrophages for the systemic and additional human enterocyte cells for the colonic inflammation, cytokine releases were aimed to be detected as responses for the inflammatory conditions. Wound healing activity was also investigated *in vitro* by using mouse fibroblast cell line to test the proliferative activity of the relatively lower concentrations of the golden thistle extracts. Lastly, higher concentrations of the extract

mixture were applied on human colon carcinoma cell line for the cytotoxic activity on colon cancer cells *in vitro*. Throughout the following chapters, brief information about the topics, detailed methodologies and the corresponding results were given. In the last chapter, an overall conclusion for the screened properties were summarized.

CHAPTER 3

COMPOSITION AND PHYTOCHEMICAL COMPOUNDS OF *Scolymus hispanicus* L.

The golden thistle is a wild edible plant with three parts: leafy aerial, edible root bark, and rigid root internal. Recently, the golden thistle was started to be cultivated by the Turkish General Directorate of Agricultural and Research Policies in Menemen, İzmir. The dry powdered form of each plant part was utilized as a material in this thesis study. Therefore, the first aim of the study was to conduct proximate and phytochemical analyses before *in vitro* experimental models. The results of this chapter showed that the aerial sample powder had relatively higher amounts of moisture, insoluble fiber, total lipids, and ash contents with 7.11, 8.01, 23.36, 1.88, and 1.67%, respectively. The protein content was also determined as the highest among all sample powders tested, and the protein content of the aerial powder was detected as 8.01 mg/g of powder in bovine serum albumin equivalents. On the contrary, soluble carbohydrate content was the highest in the edible root bark sample as 1.728 mg mannose equivalents per gram of sample powder. When the proximate analyses were completed, hydromethanolic extracts from each plant part were obtained for further analyses. Like most of the proximate analyses, the aerial extract showed relatively higher amounts of total phenolic and flavonoid contents among all samples with 133.03±16.04 µmol gallic acid and 21.94±0.37 µmol catechin equivalents for total phenolic and flavonoid contents, respectively. However, no tannins were determined in extracts. The first phytochemical screening part of the study indicated that the hydromethanolic extracts prepared for further test tube and *in vitro* methodologies contained phenolic compounds and possibly other molecular groups to be applied in different experimental models.

3.1. Wild Edible Plants and Their Phytochemical Compositions

Wild edible plants for human nutrition are generally investigated regarding their phytochemical compositions by obtaining extracts and/or isolated with various solvents. These investigations usually target the pharmaceutical sciences for the most efficient isolation of an active compound. On the other hand, as they are being utilized for culinary purposes in raw form, the nutritional composition of these plants is also important in screening studies. According to Panfili et al. (2020), more than 100 million people in European Union countries consume wild edible plants. This data does not include other Mediterranean countries that are not union members, such as Turkey and Algeria. Recently, a demand for a healthy lifestyle and unprocessed plant products cultivated and as wild edible forms has been growing. The proximate analyses for various cultivated products have been reported in the literature. However, the nutritional composition studies are rare compared to phytochemical composition studies for wild edible plants. These wild edible plants might be sources of especially fiber and specific minerals.

Golden thistle (*S. hispanicus* L.) is a wild edible plant recently cultivated in its native region of the Mediterranean. It is used in traditional recipes of Mediterranean populations in different countries. The main edible part of the golden thistle is its root bark with some of the aerial leaves in Turkish cuisine. Yet, the rigid root internal and the thorny whole aerial parts might also have some phytochemical compounds to benefit for pharmaceutical purposes. Therefore, an entire screening procedure for this plant might be included in the literature for further agricultural and pharmaceutical studies.

This chapter aimed to conduct proximate analysis for golden thistle samples obtained in dry powdered form from the Turkish General Directorate of Agricultural Research and Policies (Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü, TAGEM) and to investigate the overall phytochemical compounds of the extracts that have been prepared from these powdered samples.

3.2. Materials and Methods

In this section, the preparation of the golden thistle plant into powdered form was summarized first. In addition, methodologies of the proximate analyses such as moisture,

lipids, protein, available carbohydrates, insoluble fiber, and ash contents were explained in detail. Then, the extraction procedure was expressed to be used in phytochemical composition analyses, including total phenolic content, flavonoid content, tannin content, and sterol content, as well as other investigations in this overall study.

3.2.1. Materials and Chemicals Used

Coomassie blue G250 (115444), Bovine serum albumin (BSA, A9418), phenol (P4161), sodium hydroxide (06203), sodium acetate (S8750), gallic acid (G7384), sodium nitrite (31443), aluminum chloride (06620), rutin hydrate (R5143), catechin hydrate (C1251), vanillin (V1104), acetic acid (695092), hexane (296090), and p-anisaldehyde (V000479) was from Sigma brand (Mannheim, Germany). Whatman No.1 filter paper (WHA1001090), sulfuric acid (1.00063), Folin-Ciocalteu reagent (910.030), sodium carbonate (1.06392), silicagel 60 F_{254} thin layer chromatography plates (105554), and chloroform (102445) were from Merck (Darmstadt, Germany). Methanol (946.062), phosphoric acid (959.062), hydrochloric acid (932.106), and ethyl acetate (920.013) were from Isolab Chemicals (Bavaria, Germany). Lastly, mannose (D63580) was from Fluka brand (Buchs, Switzerland). Unless stated otherwise, all water mentioned throughout the experiments was ultrapure (Milli-Q®).

3.2.2. Preparation of the Golden Thistle Samples

The golden thistle samples of this overall study were obtained from TAGEM as local procurement. Plants were cultivated in Menemen region, İzmir in Turkey, to be harvested in February 2019. Harvested plants were washed and cleaned first. Then, the aerial, root bark, and root internal parts of the material were manually separated. Plant parts were dried in drying cabins at 50°C and ground into powdered form. The powders were filled in dark, amber-colored glass bottles with humectant caches placed in each bottle and transported to Izmir Institute of Technology (Figure 3.1). Samples were kept at room temperature in dark conditions for proximate analyses. On the other hand, the extracts obtained from the powdered golden thistle samples were kept in lyophilized form at -20°C.

Figure 3.1. Sample Preparation Steps. The photographs were taken while (a) cleaning and slicing before drying, (b) after drying at 50°C, and (c) after being transferred to Izmir Institute of Technology for further investigations.

3.2.3. Determination of Moisture Content

The moisture contents of powdered samples were analyzed according to the Association of Official Analytical Collaboration (AOAC 2012a). In brief, 1 g powdered samples were weighed into glass tared Petri dishes and heated at 105°C for 3 h. Then, the samples were put into a desiccator for cooling and weighed again. Both moisture and dry matter contents can be calculated interchangeably by using Equation 1 below,

$$Dry\ matter\ (\%) = \frac{M_3 - M_1}{M_2} x100 \quad (1)$$

where M_1 is the weight of the glass Petri dish, M_2 is the initial sample weight, and M_3 is the dried sample weight in grams.

3.2.4. Determination of Protein Content

The soluble protein contents of the sample powders were determined by Bradford (1976) with slight modifications in a microplate format. For the preparation of the Bradford reagent, 50 mg of Coomassie Blue G250 was dissolved in 50 mL absolute methanol, and 100 mL phosphoric acid was added. The reagent mixture was then diluted to 1 L with water and filtered through Whatman No.1 filter paper. For the procedure, 250 µL of Bradford reagent was pipetted into a 96-well plate, and 10 µL of 50 mg/mL golden thistle powders dissolved in water was added into each well. The microplate was incubated at room temperature in the dark for 5 minutes, and the absorbances were recorded at 595

nm at the last step (Multiskan GO, Thermo Scientific, Massachusetts, USA). Bovine serum albumin (BSA) was used as the standard material, and the results were given as mg BSA equivalents per gram of powder.

3.2.5. Determination of Soluble Carbohydrates

Total soluble carbohydrates of the golden thistle powders were determined according to Masuko et al. (2005) with the phenol-sulfuric acid method. The samples are first dissolved in water to a concentration of 100 µL. 50 µg/mL of each sample was then mixed with 150 µL of concentrated sulfuric acid and 30 µL of 10% phenol solution. Then, the samples were incubated in a 90°C water bath for 5 minutes and cooled for the following 5 minutes. At the end of the experiment, the absorbances were recorded at 490 nm. Mannose was used as the standard for this experiment, and the results were given in mmol mannose/g sample.

3.2.6. Determination of Crude Fiber and Ash Contents

The crude fiber content of the sample powders was determined with the standard procedure of the AOAC (2012b) gravimetric method. In brief, 2 g from each sample powder was weighed, and 200 mL of 0.128 M sulfuric acid was added. The reaction mixture was boiled for 30 minutes with continuous stirring, followed by filtration through a cheesecloth. The residue that remained in the cheesecloth was washed with 500 mL of hot water and collected with 200 mL of 0.313 M sodium hydroxide solution. The new reaction mixture was again boiled for 30 minutes with continuous stirring and washed with 500 mL of hot water at the end of the period. The insoluble fiber content was collected in a tared crucible, dried at 130°C for 2 hours, cooled, and weighed. Lastly, to determine the ash content, the crucibles of each sample were burned at 550°C for 2 hours, cooled, and weighed. When all weighs were collected, crude fiber content was calculated by using Equation 2 below,

$$Crude\ Fiber\ (\%) = \frac{W_1 - W_2}{W_s} x100 \quad (2)$$

where W_1 is the sample weights after drying, W_2 is the sample weights after burning, and W_s is the sample weights at the beginning of the experiment. As the ash content was weighed in this experiment, the data was used without additional experiments. The ash content in percentage was calculated according to Equation 3 below,

$$Ash\ (\%) = \frac{W_2 - W_c}{W_s} x100 \quad (3)$$

where W_2 is the sample weights after burning, W_c is the taring values for the crucibles, and W_s is the sample weights at the beginning of the experiment.

3.2.7. Determination of Total Lipids

Total lipids in the sample powders were determined with some modifications in the gravimetric method of Phillips et al. (1997). Briefly, an extraction mixture of chloroform, methanol, and water was prepared in a 1:2:0.8 ratio, respectively. 0.5 g from each powder was mixed with 3.5 mL of 0.5 M sodium acetate and 12 mL of extraction mixture. The reaction mixture was then incubated in an orbital shaker for 2 hours at 125 rpm. After adding 4 mL of chloroform, the mixture was again incubated in the orbital shaker for 30 minutes at 125 rpm. Then, 4 mL of water was added, followed by another shaking at the same period and conditions. The extracts were centrifuged at 2300 rpm for 10 minutes in the next step. The tubes were rested for equilibrium for the following 15 minutes, and the chloroform layer was taken into tared glass tubes. The tubes were kept at 60°C water bath with opened caps and in a fume hood until all the chloroform layer was evaporated. In the last step, the tubes were taken into a 101°C oven to remove the remaining chloroform for 30 minutes, cooled, and weighed. Total extractable lipid content in percentage was then calculated with Equation 4 below,

$$Lipids\ (\%) = \frac{W_2 - W_1}{W_3} x100 \quad (4)$$

where W_2 is the wights of the glass tubes after complete removal of chloroform, W_1 is the counterweights of glass tubes, and W_3 is the sample weights at the beginning of the extraction.

3.2.8. Extraction

Hyrdomethanolic extracts from each sample powder were obtained for further experiments with slight differences. 10 g of each sample was mixed with 90% methanol in a socketed glass container with a magnetic stirrer. A water bath for more effective heat transfer was formed in an apparatus prepared for extraction steps (Figure 3.2). There was a thermocouple and cooler attached to the system while the water bath contained was heated to the desired temperature. The root bark and root internal samples were extracted at 80°C, and the aerial sample was extracted at 40°C for 16 h at the first step. Then, the extract mixtures were centrifuged at 700xg for 5 minutes for a mild separation of solid particles and liquids. The supernatants were filtered, and the remaining pellets were re-extracted with water for an additional 4 h at their corresponding temperatures in the same extraction apparatus. Similar centrifugation and filtering steps were applied to the water extracts, and at the last step, the methanolic and water extracts were mixed.

Figure 2.2. The Apparatus Used for the Extractions of Golden Thistle Samples

When the hydromethanolic extract mixture was obtained, the methanol fraction was evaporated at 40°C under vacuum, and the remaining water was freeze-dried. The lyophilized powders were aliquoted in microcentrifuge tubes sealed with sealing films (Parafilm®) to prevent moistening, and each aliquoted tube was utilized for an experiment and discarded throughout the experimental procedures.

3.2.9. Thin Layer Chromatography (TLC)

The golden thistle hydromethanolic extracts were experimented with thin layer chromatography for a general estimation of phytochemical compounds with three different solvent mixtures. The extracts were applied to a Silicagel 60 F_{254} type TLC plate with solvent systems of different polarities. In the first one, ethyl acetate, acetic acid, formic acid, and water has been used in 100:11:11:27 ratios. Additionally, chloroform, methanol and water system in 60:40:10 ratios were also experimented. After the applications, the plates were dyed using 20% sulfuric acid solution and heated until detecting visible compound bands.

3.2.10. Determination of Total Phenolic Content

Total phenolic contents of the golden thistle extracts were determined with the procedure of Rufino et al. (2010). 50 µL of each sample dissolved in water was added to 250 µL 10% (v/v) Folin-Ciocalteu reagent and incubated at room temperature in the dark for 5 minutes. Then, 200 µL of 7.5% (w/v) sodium carbonate was added, and the samples were kept in the dark at room temperature for 1 h. At the end of the period, the absorbances were recorded at 760 nm. Gallic acid was used as the standard, and the results were given in µmol gallic acid equivalents (GAE) per g of extract.

3.2.11. Determination of Flavonoid Content

Flavonoid contents of the extracts were detected according to Marmouzi et al. (2017). 50 µL of each sample was mixed with 200 µL of water and 5 µL of 5% (w/v) sodium nitrite.

The reaction mixtures were kept at room temperature in the dark for 5 minutes. Then, 15 µL of 10% (w/v) aluminum chloride was added, and the samples were again kept in the dark at room temperature for an additional 6 minutes. After adding 100 µL of 1 M sodium hydroxide and 120 µL of water, the last incubation step at the same conditions was applied for 30 minutes. The absorbances were recorded at 510 nm at the end of the experiment. Both catechin and rutin were used as the standard materials, and the results were given in µmol standard equivalents for each gram of sample.

3.2.12. Determination of Tannin Content

Tannin contents of each sample were also determined according to the procedure given in Marmouzi et al. (2017). 25 µL of samples were added into 750 µL of 4% (w/v) vanillin reagent prepared in methanol and shaken vigorously. After adding 375 µL of concentrated hydrochloric acid, the samples were incubated at room temperature for 20 minutes and the absorbances were recorded at 500 nm. Catechin was used as the standard for this experiment and the results were given in catechin equivalents per gram of extract.

3.2.13. Statistical Analyses

All data were given as mean±standard deviation for at least three experiments ($n \geq 3$). The samples were compared by using one-way analysis of variance (ANOVA). Tukey's post hoc test was used for result comparisons ($p<0.05$).

3.3. Results and Discussion

At the beginning of this thesis study, proximate analyses were applied to the golden thistle powders to estimate the moisture, protein, soluble carbohydrates, insoluble fiber, total lipid, and ash contents of the sample powders. On the other hand, as the focus of the study was to evaluate the effects of phytochemical compounds of the golden thistle plant, a crude extract of each plant part was prepared and evaluated for their total phenolic, flavonoid, and tannin contents.

3.3.1. Proximate Analyses on the Golden Thistle Powders

The summarized results of the proximate analyses are given in Table 3.1. As the samples are provided as powders, the moisture contents were relatively low. The aerial powder resulted in the highest moisture content, while root bark and root internal powders followed ($p<0.05$). In literature, the moisture content for golden thistle powder was reported as 8.53±0.8% (Altiner and Sahan 2016), which is slightly different than the root bark powder results of this study.

The powders were dissolved in water as 10 mg/mL for the protein contents. In addition, a mixture sample was constituted by mixing the three dissolved samples in equal amounts. Then, a Bradford protein content assay was conducted to determine the soluble protein contents. The results indicated that the aerial sample had the highest protein content in bovine serum albumin (BSA) equivalents, followed by root internal, mixture, and root bark, respectively ($p<0.05$). Interestingly, although the root bark is considered as the edible part of the golden thistle plant, it had the lowest protein content of all. On the other hand, it should also be noted that these results might change when the edible and inedible portions were subjected to physiological conditions and/or *in vitro* digestion procedures. Altiner and Sahan (2016) reported 11.07% protein content for root bark flour with the Kjeldahl method, which detects the total nitrogen amount. Even though the comparison between the results of the two studies is contentious due to the difference between the methods, the protein content of the root bark powder in this study corresponds to approximately 4.19 in percentage, which is lower than that of the Kjeldahl method. The Kjeldahl method detects not only protein and peptide contents but also other nitrogenous compounds, while the Bradford reagent binds with lysine and arginine amino acids in principle (Anal and Koirala 2018). Therefore, the Bradford assay protein content results are logically lower than the Kjeldahl assay result.

As the powders were dissolved in water for proximate analyses, the modification of the DuBois et al. (1956) method by Masuko et al. (2005) applied in this study represents the soluble carbohydrate contents of the golden thistle powders. Differently from the protein content results, soluble carbohydrates were lowest in the aerial part of the golden thistle powders ($p<0.05$). On the contrary, root bark, root internal, and mixture extracts gave 1.3-1.7 mg mannose equivalents/g powder range of soluble carbohydrates, which are not significant in between ($p>0.05$). Similar to the protein content results, in literature, 1.14-

9.24% total carbohydrate content was reported for the wet weight of the golden thistle plant (García-Herrera et al. 2014). As the two methodologies applied for determining carbohydrates are different, the results might be considered incomparable in that manner. In addition to soluble carbohydrate content, the insoluble fiber content of the powders was also determined. The results showed significant differences between the powders. The highest insoluble fiber content was detected in aerial powder ($p<0.05$). The insoluble fiber content of the aerial powder was 5.3 and 2.5-folds higher than that of root bark and internal powders, respectively. This high insoluble fiber result of the aerial part may be due to the presence of lignin in the structure. It is well-known that lignin is an insoluble fiber and provides rigidity to plant structure (Claye, Idouraine, and Weber 1996, Weng and Chapple 2010). Additionally, the presence of lignin in other members of the Asteraceae family has also been reported (Meiri and Dulberger 1986, Nguyen et al. 2021). Therefore, it is likely that the thorny structure of golden thistle aerial leaves may contain lignin and other insoluble fibers. On the contrary, the insoluble fiber content for the edible root bark and rigid root internal parts was not as high as the aerial part. Yet still, the fiber content of the rigid root internal was 2.1 times higher than the relatively soft structure of the root bark, as expected.

Table 3.1. Proximate Analyses Results*.

Sample	Moisture Content (%)	Protein Content (mg BSAE/g powder)	Soluble Carbohydrate Content (mg ME/g powder)	Crude Fiber Content (%)	Total Lipids (%)	Ash Content (%)
Aerial	7.11±0.21[a]	8.01±0.88[a]	0.163±0.02[a]	23.36±1.44[a]	1.88±0.36[a]	1.67±0.31[a]
Root Bark	5.32±0.29[b]	4.19±0.63[b]	1.728±0.15[b]	4.41±0.23[b]	0.45±0.13[b]	0.51±0.17[b]
Root Internal	4.22±0.31[c]	7.31±1.84[a]	1.661±0.27[b]	9.51±0.25[c]	1.00±0.43[b]	N.D.[c]
Mixture[**]	N.A.	7.18±0.82[a]	1.316±0.18[b]	N.A.	N.A.	N.A.

[*]The results are given as mean±standard deviation. Different superscript letters indicate the statistical significance of one-way ANOVA with Tukey's post hoc test in each column. [**]Mixture sample represents a mixture of each dissolved sample in equal volumes. BSAE: Bovine Serum Albumin Equivalents, ME: Mannose Equivalents, N.A.: Not Applied.

For the last proximate analysis, the total lipids and ash contents of the sample powders were determined in the same gravimetric experiment. However, the total lipids for all

sample powders were relatively low, in a range of 1.00 to 1.88%. The highest lipid content was detected in aerial powder, while the root bark powder gave the lowest total lipids ($p<0.05$). The root internal powder had no significantly different total lipids from root bark ($p>0.05$). The lipid content of root bark powder was very similar to the 0.45% lipids reported by Altiner and Sahan (2016) in their work. As the total lipids, experimental procedure included the determination of ash content for subtraction, the ash content determined for total lipids analysis was also utilized as individual results. Similar to the other proximate analyses results, the highest ash content was detected in aerial powder ($p<0.05$). On the other hand, the ash content reported in the literature for the root bark portion was different than the results obtained from this experiment, with a value of 4.74% (Altiner and Sahan 2016).

3.3.2. Overall Phytochemical Components of the Golden Thistle Hydromethanolic Extracts

The golden thistle powders were relatively high in insoluble fiber compared to macronutrients. In addition, the scope of this current study was to examine the effects of phytochemical components present in the golden thistle plants. Therefore, an extraction step was included to obtain more concentrated phytochemical groups to be tested on cell culture models in further experiments. To have a preliminary information for the extract components, the extracts were subjected to thin layer chromatography by using two different mobile phases (Figure 3.3). The first mobile phase of ethyl acetate, acetic acid, formic acid, and water gave more obvious separation among the two systems, indicating polar characteristics of the compounds present. In addition, chlorogenic acid standard (CGA) was included in the TLC applications and both 365 nm and visible light bands showed the presence of chlorogenic acid, especially in the Aerial extract (Figure 3.3a). Also, intense dark colored-bands in the bottom of the Root Bark and Root Internal applications showed the presence of saccharides in these extracts. In Figure 3.3b, chlorophyll in the Aerial extract was detected as the red band in the 365 nm illustration. Overall TLC assay for the individual extracts revealed that relatively polar compounds could be obtained from the hydromethanolic extraction.

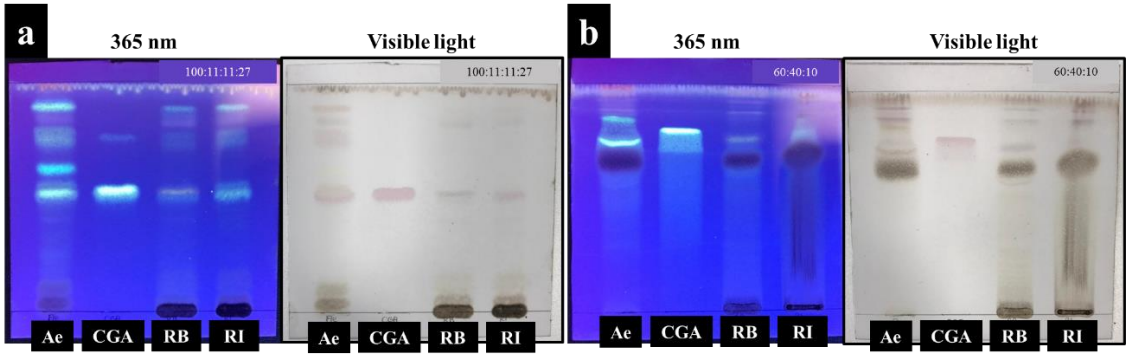

Figure 3.2. Thin Layer Chromatography (TLC) Results of the Extracts. The three individual extracts of the golden thistle plant were examined in two different mobile phases with different polarities. (a) The more-polar solvent system of ethyl acetate, acetic acid, formic acid, and water in 100:11:11:27, and (b) The less-polar solvent system of chloroform, methanol, and water in 60:40:10 ratios. After applications, the TLC plates were sprayed with 20% sulfuric acid solution and subjected to heat for band visibility. Illustrations are given as photographs taken under 365 nm and visible daylight. Ae: Aerial, CGA: Chlroogenic Acid, RB: Root Bark, RI: Root Internal.

The total phenolic and flavonoid contents of the extracts and mixture were given in Table 3.2. The aerial extract showed the highest total phenolic content in all samples ($p<0.05$). The total phenolic content determined for the aerial extract was 3.4, 2.6, and 1.6-folds higher than root bark, root internal, and mixture samples, respectively. The root bark extract was determined as the lowest total phenolic content of all samples. In addition, the flavonoid contents of the extracts were determined and calculated for two different standards.

Table 3.2. Phytochemical Compositions of Golden Thistle Hydromethanolic Extracts*.

Sample	**Total Phenolic Content**	**Flavonoid Content**	
	(µmol GAE/g extract)	**(µmol CE/g extract)**	**(µmol RE/g extract)**
Aerial	133.03±16.04[a]	21.94±0.37[a]	13.58±0.22[a]
Root Bark	39.24±1.00[b]	2.61±0.15[b]	1.98±0.09[b]
Root Internal	51.32±1.20[b]	5.46±0.16[c]	3.70±0.09[c]
Mixture**	81.42±5.69[c]	10.09±0.53[d]	6.47±0.32[d]

*The results are given as mean±standard deviation. Different superscript letters indicate the statistical significance of one-way ANOVA with Tukey's post hoc test in each column. **Mixture sample represents a mixture of each dissolved sample in equal volumes. GAE: Gallic Acid Equivalents, CE: Catechin Equivalents, RE: Rutin Equivalents.

Like total phenolic content results, the highest flavonoid content was detected in aerial extract ($p<0.05$). Such a result was expected as flavonoid-type compounds are a subset of all phenolic compounds present in a plant (Vermerris 2006). On the contrary, no significant tannin content was detected in the extracts. Therefore, the results of the tannin content assay were not included in Table 3.2.

The total phenolic contents reported by Marmouzi et al. (2017) for the same parts of absolute ethanol extractions were lower than the total phenolic contents of the current study. The possible explanation for this differentiation between the same plant could be the growing and extraction conditions. The phenolic compounds are secondary metabolites of plants in their defense mechanism. Therefore, they might differ according to the stress types and magnitudes that the plant was exposed to (Dixon and Paiva 1995). In addition, the extraction conditions between the two studies compared are different. It was stated that combining two or more solvents in the phenolic compound extraction step might result in various constituents when compared to a single solvent system due to polarity (Alara, Abdurahman, and Ukaegbu 2021). Thus, different extraction conditions might also result in different phenolic content values for the same plants.

3.4. Conclusion: Composition of the Golden Thistle

In conclusion, the proximate analyses results showed that the aerial powder had relatively higher amounts of protein, insoluble fiber, total lipids, and ash contents when compared to root bark and root internal sample powders. On the other hand, the highest soluble carbohydrate content was detected in the root bark sample, which is the traditionally consumed part of the golden thistle plant. For the phytochemical composition, hydromethanolic extracts from each sample powder were obtained. Among the extracts, the highest total phenolic, as well as flavonoid contents, were detected in the aerial extract. No tannin was detected in the extract samples. According to the first screening results of the study, phenolic-type phytochemical components were included in the freeze-dried extracts to be further studied in both test tube and cell culture models *in vitro*.

CHAPTER 4

ANTIRADICAL ACTIVITIES OF THE *Scolymus hispanicus* L. EXTRACTS

In this part, the antiradical activities of the golden thistle hydromethanolic extracts were investigated. For that purpose, activities against ABTS and DPPH radicals were determined. In addition, Ferric Reducing Antioxidant Power (FRAP) assay was also utilized. The overall results showed that the aerial extract possessed the most potent antiradical activity for all three assays ($p<0.05$). The aerial extract showed 0.285±0.019 μmol Trolox Equivalents (TE) per mg of the extract antiradical activity in ABTS, and 0.132±0.002 μmol TE/mg extract in DPPH assays. The same aerial extract resulted in 1.15 μmol TE/mg extract and 1.38 μmol ascorbic acid equivalents (AAE) per milligrams of the extract FRAP potency. On the contrary, both root bark and root internal extracts showed lower antiradical activities with no statistical significance ($p>0.05$). The antiradical activity shown by the aerial extract was higher than some similar studies conducted with the same material from another region and in accordance with other Asteraceae plants. In conclusion, the hydromethanolic extracts obtained from the three parts of the golden thistle plant showed moderate antiradical activity in test tube methods.

4.1. Plant Phytochemicals for Antiradical Activity

Since the relationship between urban diet and metabolic diseases, the consumer demand for more healthy and natural products has increased (Soobrattee et al. 2005). Therefore, especially plant-based foods rich in phytochemical compounds are recommended for a healthy diet. One of the most attributed health effects of these phytochemicals is their antioxidant potential. Antioxidant compounds have the ability to delay or inhibit the oxidation of an oxidizable compound at low concentrations (Halliwell 1995). This antioxidant activity is generally linked to the presence of phenolic compounds in all phytochemical research. Phytochemical compounds can boost the self-antioxidant system of the body and reduce the risk of various diseases such as cancer, heart diseases, and

diabetes while possessing antibacterial, antiviral, anti-inflammatory, and, finally, anti-allergenic activities (Decker 1997). Thus, phenolic-rich foods might help the body to cope with the pro-oxidant and pro-inflammatory meals meaning high fat and carbohydrates (Burton-Freeman 2010). The main mechanism of the antioxidant action of the phenolic compounds absorbed in the body is their hydrogen atom donation for radical scavenging activity. The phenolic compounds can directly interact with free radicals like superoxide, $O_2^{-\cdot}$, and NO· via their radical quenching ability (Croft 1999, Shahidi and Ambigaipalan 2015). Furthermore, there are scientific reports on the molecular effects of the phenolic compounds as well. According to Burton-Freeman (2010), phenolic compounds can act on pathways including inflammation, insulin action, platelet function, and vascular relaxation on cell signaling. In addition, the modulatory effects on mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NFκB), peroxisome proliferator-activated receptor gamma (PPARγ), protein kinase C, etc. have been reviewed (Soobrattee et al. 2005).

In food and phytochemical compound research, antioxidant activity is often investigated mainly through *in vitro* methods. In these assays, the ability of an antioxidant compound to break the chain reaction of oxidation is utilized in terms of free radical scavenging activity. On the other hand, these protocols generally fail to satisfy the biochemical, metabolic, and physiological conditions of the body (Martins, Barros, and Ferreira 2016). Therefore, critics on the test tube antioxidant methods are present. Halliwell (1995) states that the concentrations used in these assays should be similar to the *in vivo* conditions, and the radicals used should be biologically relevant. Although the best approach to study the antioxidant activity is achieved by animal and clinical trials, the test tube methods still contribute to the investigations as a preliminary insight. There are widely studied methodologies for the determination of radical scavenging activity. However, naming these methods as *antiradical activity* rather than *antioxidant activity* might be another option (Tirzitis and Bartosz 2010).

This chapter aimed to determine the antioxidant activity of golden thistle hydromethanolic extracts using Trolox equivalent antioxidant capacity (TEAC), scavenging activity of DPPH radical, and ferric reducing antioxidant power (FRAP) assays.

4.2. Materials and Methods

This section shows three widely applied antiradical activities as Trolox equivalent antioxidant capacity (TEAC) method using $ABTS^{2-}$ radical, determination of DPPH radical scavenging activity, and ferric reducing antioxidant power (FRAP) are investigated for the golden thistle extracts. The ABTS and DPPH assays can be considered relatively similar in principle as they both detect the decolorization of a stable radical. Yet, ABTS assay is more often used for water-soluble substances and extracts, while DPPH assay might be more useful for extracts with organic solvents. Additionally, the FRAP assay that determined the reduction of the ferric salt is applied in acidic conditions. Therefore, using more than one antiradical activity assay is often recommended, especially for raw extracts and screening studies.

4.2.1. Materials and Chemicals Used

Potassium persulfate (379824), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 238813), 2,2-Diphenyl-1-picrylhydrazyl (DPPH, D9132), acetic acid (695092), sodium acetate (W302406), and iron (III) chloride (157740) were obtained from Sigma (Mannheim, Germany), 2,3,5-Triphenyltetrazolium chloride (TPTZ, 1.08380), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 10102946001), and L-ascorbic acid (33034H) were from Merck (Darmstadt, Germany), Roche (Basel, Switzerland), and Honeywell-Fluka (North Carolina, USA), respectively. For phosphate-buffered saline (PBS), ready-to-use PBS tablets from Sigma brand (524650) were used according to the product descriptions. Unless stated otherwise, all water mentioned throughout the experiments was ultrapure (Milli-Q®).

Throughout this chapter, hydromethanolic extracts of three individual parts of the golden thistle plant aerial, root bark, and root internal were used as well as a mixed extract of equal volumes from each.

4.2.2. Trolox Equivalent Antioxidant Capacity (TEAC)

The Trolox equivalent antioxidant capacity assay was applied according to the procedure of Re et al. (1999). First, 7 mM ABTS and 2.45 mM of potassium persulfate solutions were mixed in equal volumes and kept in the dark with continuous stirring for 16 h. Next, the radical solution was diluted with phosphate-buffered saline to 0.7 absorbance before the assay. 1 mL of this reaction medium was mixed with 1-20 µL of sample varying for each sample at different amounts and incubated at room temperature for 5 minutes. Lastly, absorbances were recorded at 734 nm. Trolox was used as the standard antioxidant compound for this assay in 0.25-10 mM concentrations and 5 µL amount. The inhibition percentages were calculated by using Equation 5 below,

$$Inhibition\ (\%) = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} x100 \quad (5)$$

where Abs_{blank} is the absorbance recorded at the beginning of the experiment, and Abs_{sample} is the absorbance recorded for the sample after 10 minutes of incubation. Then, a linear plot was obtained with inhibition (%) versus sample amount. The slopes from each replicate were obtained from these sample plots. A similar plot was also obtained for the standard Trolox with concentration on the x-axis, and the slope for the Trolox standard was also obtained. In the last step of the calculations, standard slope was divided by the sample slopes in terms of µM Trolox/µL sample.

4.2.3. DPPH Radical Scavenging Activity

The scavenging activity of DPPH radical for the golden thistle extracts was applied according to the method of Blois (1958) and Martinez-Morales et al. (2020) in modifications and calculations. For that, first 460 µM DPPH radical was diluted from 900 µM of stock solution in methanol. For the assay, a 50 µL extract sample was added to 200 µL of 460 µM DPPH radical and incubated at 30°C for 20 minutes. At the end of the period, absorbances were recorded at 517 nm. For determining the 50% inhibitory concentration (IC_{50}), first nanograms of the sample in the assay (nI) were calculated with Equation 6.

$$nI = C\ x\ V\ x\ F \quad (6)$$

where C is the concentration of the sample in µg/mL, V is the volume of the sample in mL, and F is the conversion factor from µg to ng. In the second step of the calculations, nanomoles of the DPPH (nD) was calculated using Equation 7.

$$nD = M\ x\ V\ x\ F \quad (7)$$

where M is the radical concentration in molars, V is the volume of the radical in liters, and F is the conversion factor from mole to nanomole. A standardized unit was obtained by dividing nI to nD in ng antioxidant/nmol DPPH. In addition, the inhibition percentage was calculated by using Equation 5. Lastly, a nonlinear plot was obtained with inhibition (%) versus the logarithm of the standard unit on GraphPad Prism software. The IC_{50} value of the plot was extracted for each sample replicate and the standard Trolox for the final DPPH radical scavenging activity.

4.2.4. Ferric Reducing Antioxidant Power (FRAP)

Ferric Reducing Antioxidant Power (FRAP) assay was conducted according to the procedure of Benzie and Strain (1996). An acetate buffer at pH 3.6 was prepared using 0.1 M sodium acetate and 0.1 M acetic acid. Briefly, 46.3 mL of acetic acid and 3.7 mL of sodium acetate were mixed, and the pH was adjusted to 3.6 with either 3 M of hydrochloric acid or sodium hydroxide. Then, 10 mM TPTZ and 20 mM ferric chloride solutions were prepared. For the FRAP reagent, acetate buffer, TPTZ, and ferric chloride were mixed in a 10:1:1 ratio, respectively. 180 µL of this FRAP reagent was mixed with 20 µL of extract sample and incubated at room temperature for 24 minutes. Lastly, the absorbances were recorded at 593 nm. Both Trolox as well as L-ascorbic acid were used as the standards for this assay, and the results are given in µmol ascorbic acid equivalents (AAE) per gram of extract.

4.2.5. Statistical Analyses

All data were given as mean±standard deviation for at least three experiments (n≥3). The samples were compared by using one-way analysis of variance (ANOVA). Tukey's post hoc test was used for result comparisons ($p<0.05$).

4.3. Results and Discussion

Determination of antioxidant or antiradical activity provides an insight into the unknown molecular groups before further investigations. Yet, there might be differences between the antioxidant capacity and other activities that any crude extract or isolated molecular groups have. Therefore, although conducting an antioxidant capacity method might be helpful at the beginning of a study, it is not certain that the positive activity would possess other *in vitro* and/or *in vivo* effects. It is also crucial to conduct more than one protocol regarding antioxidant capacity as methods that determine the antiradical activity differ in mechanism of action, especially in test tube methods.

Table 4.1. Antiradical Activities of the Golden Thistle Extracts*

	TEAC	**DPPH**		**FRAP**
	µmol TE/mg sample	µmol TE/mg sample	IC_{50} in g/mL	µmol AAE/mg sample	µmol TE/mg sample
Aerial Extract	0.285 ± 0.019^{a}	0.134 ± 0.002^{a}	5.27 ± 0.60^{a}	1.15 ± 0.06^{a}	1.38 ± 0.07^{a}
Root Bark Extract	0.102 ± 0.001^{b}	0.027 ± 0.001^{b}	77.96 ± 14.81^{b}	0.21 ± 0.003^{b}	0.25 ± 0.004^{b}
Root Internal Extract	0.090 ± 0.002^{b}	0.048 ± 0.002^{c}	58.59 ± 9.74^{b}	0.31 ± 0.05^{b}	0.36 ± 0.06^{b}
Extract Mixture	0.182 ± 0.008^{c}	0.102 ± 0.001^{d}	16.38 ± 0.89^{a}	0.73 ± 0.09^{c}	0.87 ± 0.11^{c}

*Different superscripts indicate statistical significance between the samples in each column according to one-way Analysis of Variance with Tukey's post hoc test. TE: Trolox Equivalents, AAE: Ascorbic Acid Equivalents

The antiradical activities of golden thistle crude extracts are given in Table 4.1. If the Trolox Equivalent Antioxidant Capacity (TEAC) values for the inhibition of ABTS radical are evaluated, the highest TEAC value was found in the Aerial extract ($p<0.05$). On the other hand, root bark and root internal samples resulted in relatively lower TEAC

values, indicating less antiradical activity for ABTS radical. The extract mixture sample consisted of equal volumes of each extracted sample, therefore, a value between the lowest and the highest TEAC values was as expected.

Scavenging activity of DPPH radical was the second antiradical activity method conducted. The results were similar to the TEAC experiment for the Aerial sample, with one of the highest Trolox Equivalent radical scavenging activity among all samples ($p<0.05$). On the contrary, both root samples of bark and internal extracts showed significantly lower DPPH antiradical activity when compared to Aerial or extract mixture. It is probable that the presence of Aerial extract in the mixture resulted in higher antiradical activity. As the IC_{50} value indicates the inhibitory concentration of a sample for 50% reduction, lower IC_{50} values for higher antiradical capacities should be expected. Therefore, the IC_{50} values followed an ascending trend in the order of Aerial, extract mixture, Root Internal, and finally Root Bark samples, with no significance between the root samples ($p>0.05$). The IC_{50} determined for the Aerial extract (leaves) was lower than the hot boil aqueous extract of Berdja et al. (2021). The authors reported higher antiradical activity for their aqueous extract from their experimental control. The presence of stems, as well as the extraction conditions, could result in higher DPPH antiradical activity. In terms of DPPH antiradical activity, there are also some other researches reporting the activity in percentage of inhibition (Abu-Lafi et al. 2019, Pieroni et al. 2002). However, these results could not be considered as comparable due to the difference between the beginning concentration of DPPH radical.

Ferric Reducing Antioxidant Power (FRAP) was the last procedure applied to the golden thistle extracts. L-ascorbic acid is the standard used in the FRAP assay. However, to see the trend, both ascorbic acid and Trolox were used in the FRAP assay, and the results were different than those of other antiradical assays. Both results were close to each other and similar to the overall antiradical activity results. The highest FRAP was observed in Aerial extract, followed by extract mixture, Root Internal, and Root Bark extracts, respectively. In addition, antiradical activities for the three procedures are summarized in Figure 4.1 only in Trolox equivalents.

Antiradical capacities, as well as other phytochemical investigations, have been conducted for golden thistle extract by Marmouzi et al. (2017). The data was given in different units, yet when the conversion is applied, it can be said that the antiradical activities that the authors reported are lower than in this current work. One reason for that variation could be the different extraction techniques. Marmouzi et al. (2017) extracted

the plant in absolute ethanol for 4 hours, while the extraction of this study was applied by using both methanol and water for 20 hours in total. As the extract materials have been shown to have more polar compounds by thin layer chromatography in chapter 3 (Fig. 3.3), it can be thought that more polar compounds with antiradical activity could be extracted in this study. On the contrary, a study investigated the effects of *in vitro* digestion procedure on the phenolic content and antiradical activity of some plants, including the golden thistle (Gonçalves et al. 2019). The authors reported higher antiradical values for ABTS and DPPH antiradical activities and FRAP values.

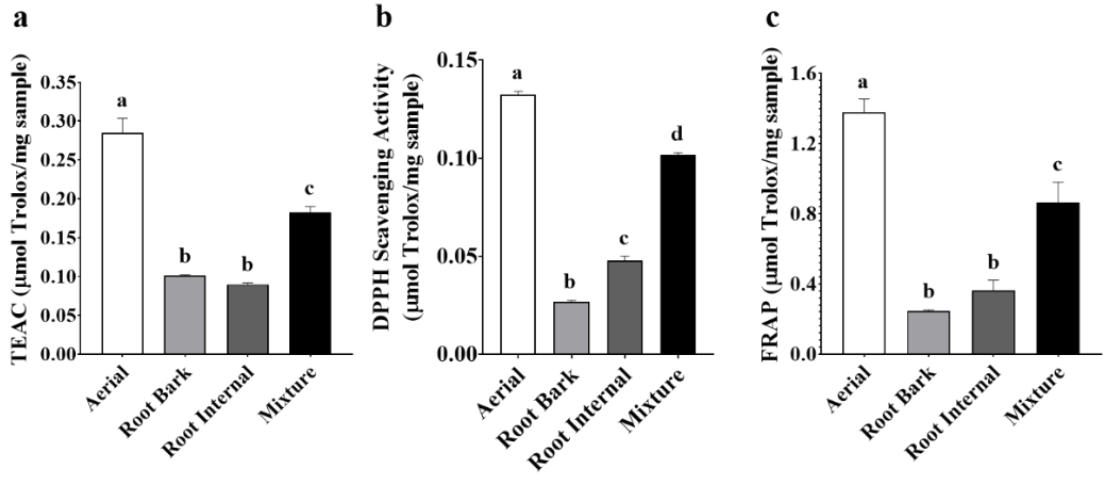

Figure 4.1. Antiradical Activities of the Golden Thistle Extracts in Trolox Equivalents. The antiradical activities of the golden thistle hydromethanolic extracts were summarized for (a) ABTS, (b) DPPH, and (c) FRAP assays. All results are given in Trolox Equivalents per milligrams of the corresponding extract. The results are given as mean±standard deviation of three experiments (n=3). Different letters above each bar represent statistical significance of one-way ANOVA with Tukey's post hoc test ($p<0.05$). TEAC: Trolox Equivalent Antioxidant Capacity, FRAP: Ferric Reducing Antioxidant Power.

In literature, some other plants from the Asteraceae family have been investigated regarding antiradical activity. For instance, *Schizogyne serica* plant native to the Canary Islands was extracted, and the antiradical activities of the extracts were investigated Caprioli et al. (2017). When the antiradical activities of the golden thistle (*S. hispanicus* L.) are compared with the *S. serica*, it can be said that the antiradical activity results of the golden thistle extracts were similar to the lower range of *S. serica* results. In another example, Jiménez et al. (2015) determined the antiradical activities of some Colombian medicinal plants, including Asteraceae members. Plants like *Clibadium surinamese* and *Pseudelephantopus spiralis* (Less.) Cronquist. showed higher values for all the assays, while others such as *Acmella brachyglossa* and *Aregatum conyzoides* L. resulted in higher

ABTS yet similar DPPH antiradical activity and FRAP value. Lastly, the results were compared to some fruits and beverages consumed for their antioxidant properties (Henning et al. 2014). For all three antiradical activity tests, the golden thistle plant showed less activity than the well-known antioxidants such as pomegranate, green tea, and grape seed. However, the antiradical activity of the golden thistle Aerial extract was higher than that of acai and goji berry.

4.4. Conclusion: Antiradical Activities of the Golden Thistle Extracts

In conclusion, golden thistle hydromethanolic extracts showed moderate antiradical activity for TEAC and FRAP methods. However, the IC_{50} values determined for DPPH scavenging activity were higher than most well-known antioxidant materials. Among the samples, the Aerial extract showed the highest potential among all three extracts in terms of Trolox equivalent ABTS and DPPH antiradical activity. Same results were also obtained for the FRAP assay. Most of the secondary metabolites, especially phenolic compounds, were detected higher in the leafy aerial part of the plant, therefore, it is probable that the determined antiradical activity might be a result of the phenolic presence in the Aerial extract.

CHAPTER 5

BIOAVAILABILITY OF CHLOROGENIC ACID IN *Scolymus hispanicus* L. HYDROMETHANOLIC EXTRACT BY AN *in vitro* MODEL

Recent evidence suggested that plant-based foods and other related products possess health-promoting activities due to their phenolic compounds. This part aimed to determine the phenolic profile of golden thistle hydromethanolic extract and the *in vitro* bioavailability of the abundant phenolic compound present. The extract mixture sample was first subjected to high pressure liquid chromatography (HPLC), and the abundant phenolic compound was detected as chlorogenic acid as 5-*O*-caffeoylquinic acid. Then, the sample was introduced to the differentiated CaCo-2 cells on a bicameral insert membrane system at 1 mg/mL concentration. After periodical sampling, the percentages of chlorogenic acid that passed through the basolateral side were determined as 0.37, 0.30, 1.13, 1.85, 3.47, and 6.94% on average for 15, 30, 60, 120, 240, and 360 minutes, respectively. The *in vitro* bioavailability trend was linear, therefore, apparent permeability was also calculated and found to be $(1.82\pm0.07)\times10^{-6}$ cm/s. The data obtained for chlorogenic acid bioavailability was in accordance with most of the literature data reported for other plant-based extracts. All in all, despite the determined chlorogenic acid bioavailability was low *in vitro*, developed formulations for enhanced bioavailability could be constituted for its health-promoting effects reported and tested *in vivo* for commercial functional foods.

5.1. Bioavailabilities of the Phenolic Compounds in Plant-Based Foods

Plant phytochemicals possess various health effects on the body. Among all phytochemicals, phenolic compounds have extensive coverage in the research area. These compounds are the secondary metabolites of the plants that are produced from phenylalanine and tyrosine as a consequence of external stress that the plant tissue experiences (Haminiuk et al. 2012, Naczk and Shahidi 2003). There are at least seven

classes of phenolic compounds, yet the classifications may vary among researchers (Fig. 5.1). The widely studied phenolic compounds include simple phenolics like phenolic acids and coumarins, and complex phenolic compounds such as flavonoids, tannins, stilbenes, lignans, and curcuminoids.

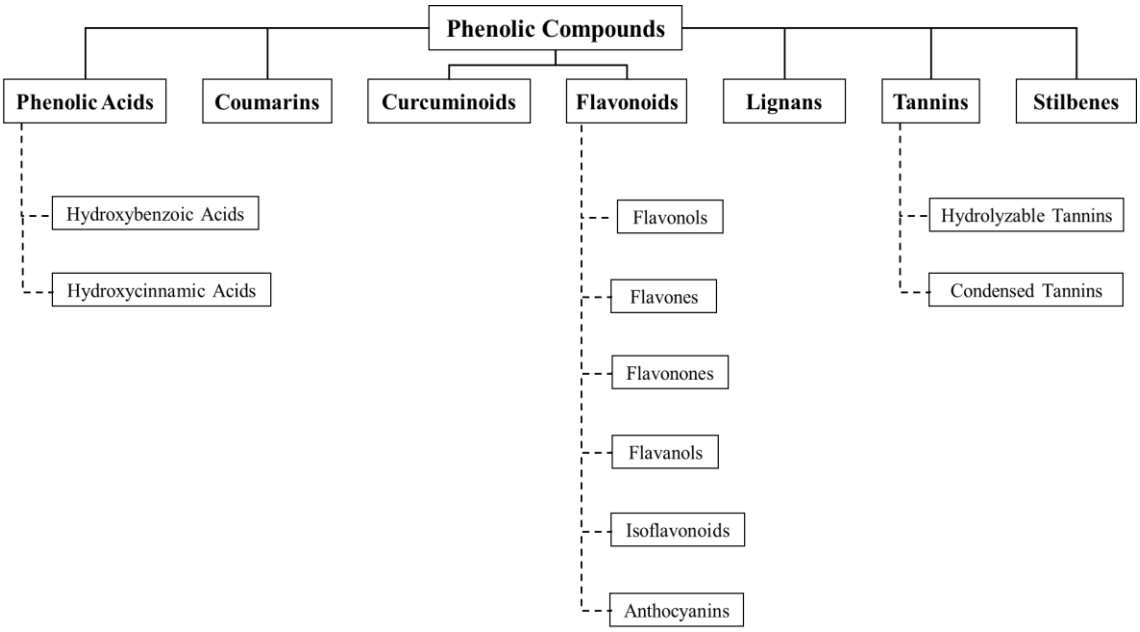

Figure 5.1. Classification of Major Phenolic Compounds in Plants

Phenolic acids are one of the largest classes of plant phenolics. This is the simplest structure of all phenolic compounds, with one phenol ring and a carboxylic acid function. According to the skeleton structures, phenolic acids with C6-C1 structures are named as hydroxybenzoic acids, while phenolic acids with C6-C3 structure are called as hydroxycinnamic acids. On the other hand, flavonoids have a C6-C3-C6 backbone with two benzene and heterocyclic pyran rings (Albuquerque et al. 2021). In a healthy and well-balanced human diet, phenolic acids and flavonoids are consumed adequately (Arruda et al. 2020). The most common sources of phenolic compounds for consumption are cereal grains, vegetables, fruits, nuts, berries (Velderrain-Rodríguez et al. 2014), with high amounts in red wine, coffee, cocoa, tea, citrus fruits, and berries (Valdés et al. 2015). Phenolic compounds are widely studied in terms of their health-promoting effects. They have been reported to have protective effects against cardiovascular diseases (Rangel-Huerta et al. 2015), anti-inflammatory action (Ambriz-Pérez et al. 2016), antioxidant, antiviral, antimicrobial, antitumor, and antibacterial properties (Haminiuk et al. 2012). The most important property of the phenolic compounds is their ability to donate an

electron to the free radical and therefore show antioxidant activity (Haminiuk et al. 2012). The radical scavenging activity of these phenolic compounds protects crucial components such as proteins, DNA, and lipids against oxidation (Albuquerque et al. 2021). Once they are absorbed in the body and reach the target tissues, they may affect gene expressions, cell signaling, and cell adhesion (Borges et al. 2010).

For the digestion, absorption, and metabolization of phenolic compounds and all other nutrients, three main concepts are very important. Sequentially, first, a compound must be released from its corresponding matrix during digestion. This digestible portion of a material is called as the *bioaccessible* portion. Factors such as peristaltic movements of the digestive system, digestive enzymes (hydrolases), and pH variations during digestion play a major role in the release that takes place throughout the gastrointestinal tract. However, in most cases, only a limited portion of the released material can be absorbed by the intestinal enterocytes. This second part of the bioaccessible portion is called as the *bioavailable* portion. Simply, it represents the amount of compounds absorbed by the enterocyte cells after digestion (Ribas-Agustí et al. 2018). Again, a limited portion of this bioavailable mass can reach the target tissue either in intact form or as a metabolite in the body. This is the smallest part of all and is called the *bioactive* portion. Because it is mandatory for a compound to reach the corresponding targeted tissue in the body, it can be said that the most important outcome is directly linked to the bioactive portion. However, research on bioactive portions includes *in vivo* studies. Generally, test-tube digestions for bioaccessible, and cell culture studies for bioavailable parts are usually conducted first before the *in vivo* animal studies or clinical trials.

The bioaccessibility and bioavailability of phenolic compounds are very important in food and nutrition research. However, the bioavailable portions of different phenolic compounds vary widely (Valdés et al. 2015). For instance, bioavailabilities for anthocyanins and isoflavones have been reported as 0.3% and 43%, respectively (Albuquerque et al. 2021, GutiErrez-Grijalva et al. 2016). The host factors that affect the phenolic compound bioavailabilities can be listed as molecular size, lipophilicity, solubility, pK_a, gastric and intestinal transit time, membrane permeability, and pH of the lumen (Naczk and Shahidi 2003), as well as molecular dimension, polymerization, the stereochemistry of the glycosidic moiety if present (Velderrain-Rodríguez et al. 2014).

During their journey, phenolic compounds first meet the human saliva providing the bitter and astringent taste (Soto-Vaca et al. 2012). Here, although the sugar-bound phenolic compound might be affected by the action of human salivary amylases, the chewing and

swallowing actions are much less effective when compared to gastric and intestinal digestions (Grgić et al. 2020). Some of the phenolic compounds are unstable at lower pH values, therefore, they might be affected during the gastric phase (Velderrain-Rodríguez et al. 2014). However, the most significant release and absorption occur in the intestinal phase. Once the phenolic compounds reach the intestine, cytosolic β-glycosidase (CBG) may act on the sugar-bound phenolic compounds and cleave the glycosidic moieties. Secondly, lactase-phlorizin hydrolase (LPH) is another enzyme that is proposed to act on phenolic compounds in the small intestine. These enzymes are the brush-border glycosidases of the small intestine (Soto-Vaca et al. 2012). The aglycone compounds, after separation from the sugar bonds, might be absorbed by passive diffusion or with the help of sodium-dependent transporters like SGLT1 from the intestinal lumen, whereas they are transported to the bloodstream via passive diffusion (Velderrain-Rodríguez et al. 2014). Monocarboxylic acid transporters (MCTs) are another route if the phenolic compound has a monoanionic carboxylic acid group and a nonpolar side chain or aromatic hydrophobic unit (Grgić et al. 2020). As stated earlier, the absorbed portion of the released and stable phenolic compounds is relatively lower. On the other hand, when these unabsorbed phenolic compounds are transported into the colon, bacterial enzymes of microflora may act on them and hydrolyze the ester bonds of phenolic compounds. Some bacteria that act on phenolic compounds are *Bacteroidetes, Clostridium, Eubacterium, Ruminococcus, Eggertbella, Lactobacillus,* and *Bifidobacteria* (Soto-Vaca et al. 2012). Hydroxycinnamic acid-type phenolic compounds generally reach the colon and become substrates of these bacterial enzymes due to the lack of esterases in the human body (Grčić et al. 2017, Faridi Esfanjani, Assadpour, and Jafari 2018, Haminiuk et al. 2012). The last absorption could take place in the colon before excretion. After absorption, the phenolic compounds are assumed xenobiotics in the body and transformed into secondary metabolites by the liver detoxification system of phase II enzymes (Velderrain-Rodríguez et al. 2014).

All in all, the bioavailability studies for phenolic compounds could be challenging, and specific research must be designed for each compound to focus on the molecular mechanism of absorption. Experimental designs, including test-tube digestions, may help to understand the effect of secreted digestive enzymes on the stability as well as the bioaccessibility of the corresponding phenolic compound. On the other hand, the bioavailable portion might also be determined by using cell culture techniques. These *in vitro* bioavailability studies could mimic the absorption performance of the human

enterocytes. As the last step, *in vivo* studies with either animal or clinical trials must be performed to unveil the portion of the phenolic compound in the targeted tissue. Generally, the secondary metabolites that are transformed by the liver in the body are believed to be more stable and effective in the targeted tissue, and these compounds are selected for the *in vivo* response of that particular experimental design (Haminiuk et al. 2012). Yet, conducting *in vivo* studies before any *in vitro* experiments for absorption might not be feasible due to the ethical and economic issues of *in vivo* studies. Especially for scanning studies of unknown molecular groups, it becomes highly important to test the bioavailable portion as well as the phenolic compound molecule types before further developments for consumption.

Therefore, the aims of this part for the golden thistle hydromethanolic extract were first to determine the type of phenolic compound present in the crude extract and to determine the *in vitro* bioavailable portion of that corresponding molecule by using a bicameral cell membrane system with CaCo-2 cell line.

5.2. Materials and Methods

For the *in vitro* bioavailability section, a bicameral cell membrane system was utilized using CaCo-2 cell line (HTB-37^{TM}) from American Type Culture Collection (ATCC$^{®}$). A cytotoxicity test was conducted to determine the possible non-toxic concentration of the golden thistle extract on the cells in addition to the experimental procedures.

5.2.1. Materials and Chemicals Used

Phosphate buffered saline (PBS) tablets (P4417), Minimum Essential Medium (MEM, M4655), non-essential amino acid solution (M7145), sodium pyruvate (S8636), penicillin-streptomycin solution (P4333), trypsin-EDTA solution (T4049), Hank's Balanced Salt Solution (HBSS, H9394), chlorogenic acid standard (PHL89175), acetic acid (695092), methanol (34860), phenol red (P3532) and 1-(4,5-Dimetylthiazol-2-yl)-3,5-diphenylformazan (MTT, M2003) were purchased from Sigma (Mannheim, Germany). Dimethyl sulfoxide (DMSO, 102952) was from Merck (Darmstadt, Germany), and fetal bovine serum (FBS) was from Gibco (Thermo Fisher Scientific,

Massachusetts, USA). 6-well inserts for bioavailability (Transwell®, 3450) were of Corning (New York, USA).

Throughout this part, a mixture sample was prepared using three individual hydromethanolic extracts in equal amounts and utilized as the main sample of the experiments at 1 mg/mL concentration. Unless stated otherwise, all water stated in the experiments was MilliQ®.

5.2.2. Cell Maintaining

CaCo-2 human adenocarcinoma cell line has been a useful *in vitro* model for permeability tests when grown and differentiated on a bicameral membrane system (Yee 1997). The cells have been grown in T-75 flasks with MEM containing 15% FBS, 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin-streptomycin solutions. All maintaining, differentiation, and experimental periods took place in a 37°C incubator with 5% CO_2. The passage numbers were kept between 30-40 throughout the experiments.

5.2.3. Cytotoxicity

As the concentration applied to the CaCo-2 cells must not have cytotoxic activity, an MTT cytotoxicity assay was conducted to determine concentration. MTT is a yellow-colored water-soluble salt that can interact with mitochondrial reductase inside the cells. When the reagent meets the enzyme, insoluble and purple-colored formazan crystals are formed. These can later be dissolved by using appropriate solvents to detect viability.

For the cytotoxicity assay, CaCo-2 cells were seeded in 4000 cell/well density in 96-well cell culture plates and incubated overnight for attachment. The next day, 1 mg/mL golden thistle extract mixture was introduced to the cells and incubated for 6 hours. At the end of the period, cell culture mediums were replaced with 100 µL fresh mediums. Then, 10 µL of MTT reagent (5 mg/mL in PBS) was added to each well and incubated for another 4 hours for the cells to form formazan crystals. The crystals were checked under a microscope, and the cell culture mediums were carefully discarded without touching the formazan. 100 µL DMSO was added to each well and pipetted for formazan solubilization, and the absorbances were recorded at 570 nm. Non-treated CaCo-2 cells

were used as the experimental control, and their absorbances determined were assumed as 100% viable. The experimental group treated with extract mixture was normalized according to the control cells.

5.2.4. Experimental Design and Transepithelial Electrical Resistance (TEER) Measurement

To evaluate the phenolic compound bioavailability of the golden thistle extract mixture *in vitro*, this model has been used in bicameral membranes (Fig. 5.2). The cells were seeded in $4.17x10^5$ cell/well density in 6-well polyethylene insert membranes and incubated overnight for attachment. The cell culture mediums were refreshed in growing and differentiation periods every other day. Once the cells were fully grown on the insert membranes and became confluent, a post-confluency period was started, and these post-confluent cells were maintained with the same cell culture medium for 21 days. During this period, the cells differentiate and form the specific structure of enterocyte cells.

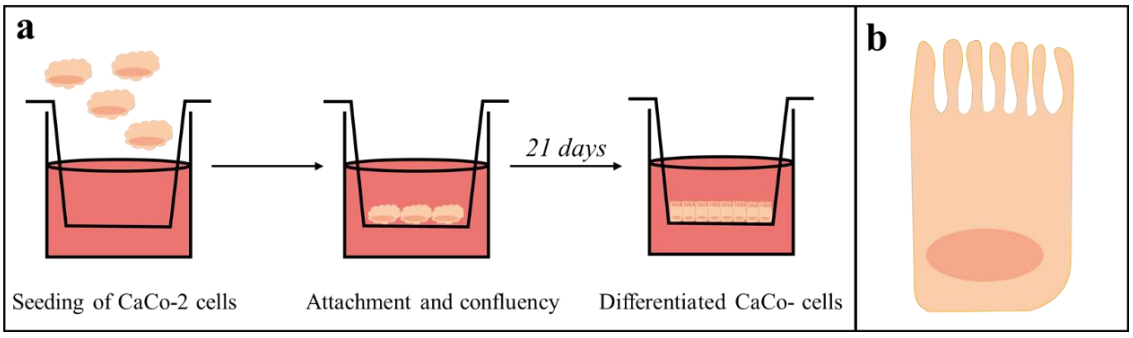

Figure 5.2. Preparation Steps for the Enterocyte-Like Structure for Absorption. (a) CaCo-2 cells were seeded, grown for full confluency, and differentiated on bicameral insert membranes for 21 days of the post-confluency period, (b) larger illustration of a differentiated CaCo-2 cell with microvilli on the apical side.

After the cells are differentiated for the specific enterocyte structure, the upper part of the membrane system represents the gastrointestinal tract that the phytochemical compounds release after digestion, called the *apical* side. On the contrary, the lower part of the plate stands for the bloodstream, where the enterocyte cells transfer the phytochemicals and other nutrients after absorption. This part is called the *basolateral* side. The cells attach to each other with tight junction proteins in the post-confluent period. In such an experimental design, the cells must remain intact during the transport assays. Therefore,

transepithelial electrical resistance (TEER) is a helpful way to check cell integrity. In this measurement, an electric current is formed between the probes, and if the cells are tightly attached, they should resist this electrical current (Fig. 5.3). Therefore, more resistance indicates better cell attachments. A typical TEER value should be at least 250 $\Omega \cdot cm^2$ (Alvarez-Hernandez, Nichols, and Glass 1991). The TEER values of differentiated cells on insert membranes were measured before and after application (World Precision Instruments, FL, USA).

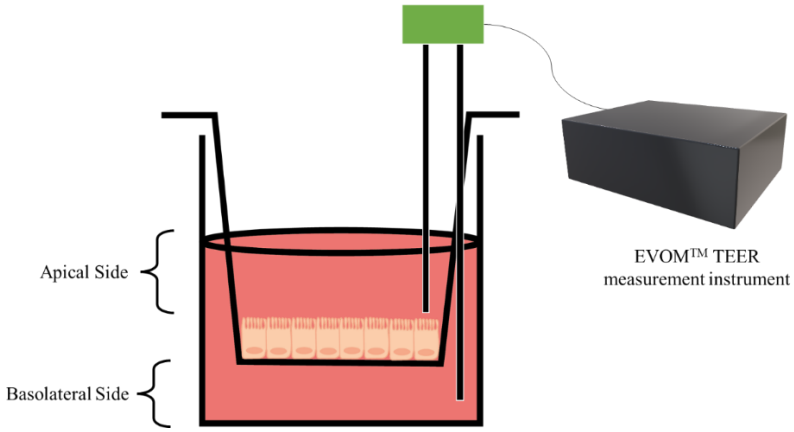

Figure 5.3.TEER Measurement. After a post-confluency period, the cells should be tightly attached. In the bicameral membrane system, the upper part is called the apical side and represents the digestive tract, while the lower part is called the basolateral side, which stands for the bloodstream, where the nutrients are transferred after absorption. The tightness of cell attachments is measured by introducing an electrical current between the apical and basolateral sides with an EVOM™ instrument that detects the resistance of the cell attachment forms. TEER: Transepithelial Electrical Resistance.

Once the cell integrity was confirmed, the golden thistle extract mixture in 1 mg/mL concentration was given in the HBSS medium to the apical side of the membrane, and the *in vitro* bioavailability study was started. Periodically, samples were taken from the basolateral side, and the phenolic compounds transported by the cells were detected in HPLC.

5.2.5. High Pressure Liquid Chromatography (HPLC) Conditions

Phenolic compounds present in the golden thistle extract medium, as well as the *in vitro* bioavailable portion, were determined with the procedure of Uncu and Ozen (2015). An

Ace C18 (Mac-Modd, Chadds Ford, PA) column was used at 35°C with gradient mobile phases A and B. Mobile phase A contained 0.2% acetic acid in the water while mobile phase B was absolute methanol at 1 mL/min flow rate. The gradient mobile phase composition is given in Table 5.1. The procedure was applied in an HPLC (Perkin Elmer, Flexar LC) with a diode array detector (DAD), in which the recordings were made at 280 and 320 nm. The injection volume was 20 μL.

Table 5.1. Gradient mobile phase used in HPLC.

Time (minutes)	**Mobile Phase A (0.2% CH_3COOH in water)**	**Time (minutes)**	**Mobile Phase A (0.2% CH_3COOH in water)**
0	90	36-37	50
0-10	70	37-38	50
10-30	70	38-42	0
30-35	60	42-46	90
35-36	60	46-60	90

5.2.6. Statistical Analyses

All data were given as mean±standard error for at least three experiments ($n \geq 3$). The samples were compared using either 2 sample t-tests or one-way analysis of variance (ANOVA), depending on the samples. For ANOVA, Tukey's post hoc test was used for result comparisons ($p<0.05$).

5.3. Results and Discussion

The results obtained before the *in vitro* bioavailability assay revealed that the experimental conditions were suitable (Fig. 5.4). 1 mg/mL of golden thistle extract mixture had no cytotoxic effect on CaCo-2 cells for 6 h of incubation, as the golden thistle extract mixture treated cells were not different than that of the non-treated control group ($p>0.05$, Fig 5.4a). In addition, TEER values measured both before and after had no statistical significance either ($p>0.05$), indicating that there were no leakages of samples from the apical to the basolateral side of the insert membrane system (Fig 5.4b).

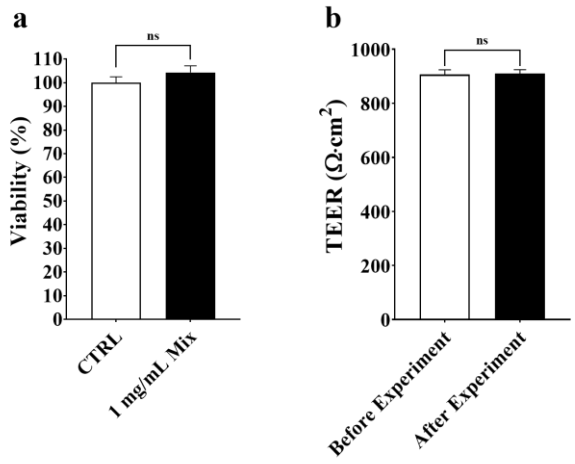

Figure 5.4. Confirmative Measurements Before *in vitro* Bioavailability Assay. (a) Cytotoxic effect of 1 mg/mL golden thistle extract mixture on CaCo-2 cells. (b) TEER measurements before and after the bioavailability assay. The results are given as mean±SEM for three replicates (n=3). Both comparisons showed no statistical significance as the results of 2-sample t-tests ($p>0.05$). CTRL: Non-treated control group, TEER: Transepithelial electrical resistance.

Next, the bioavailability study was conducted. The results for the *in vitro* bioavailability assay are given in Figure 5.5. Chlorogenic acid was the most abundant phenolic compound in the golden thistle extract mixture (Fig. 5.5a). In addition, no phenolic compound peaks were detected in the HBSS medium at the beginning of the experiment (Fig. 5.5b). On the other hand, after 6 h, there were some phenolic compounds transported to the basolateral side of the insert membranes (Fig. 5.5c). An overlay for the apical side chlorogenic acid peak at the beginning and the basolateral side chlorogenic acid peak at the end of 6 hours was also given in Figure 5.5d. If the chlorogenic acid content determined on the apical side is assumed as 100%, the contents with respect to time could be normalized accordingly. Therefore, the chlorogenic acid content during the 6 hours of *in vitro* bioavailability assay was calculated (Fig. 5.5e). The chlorogenic acid contents were not only low but also insignificantly different than each other in the first two time points of 15 and 30 minutes ($p>0.05$). The range of bioavailability was 0.37-0.30%. On the contrary, the bioavailability increased to 1.12, 1.85, and 3.47% in the following 60, 120, and 240 minutes, respectively. The bioavailabilities in these timepoints were significantly higher than their previous values ($p<0.05$). After 6 hours, the final *in vitro* bioavailability of chlorogenic acid was determined as 6.94%, which was not different from the previous 240 minutes value ($p>0.05$). Furthermore, a cell-free membrane was also used as an experimental control to confirm that the transport would be higher without

the presence of CaCo-2 cells through the insert membrane. As expected, the percentage of chlorogenic acid through the cell-free insert membrane was determined as 19.69% at the end of 6 hours. In their study, Konishi and Kobayashi (2004) reported 0.12% permeation of chlorogenic acid from apical to basolateral side after 40 minutes in pH 7.4 for both sides. Similarly, Dupas et al. (2006) reported 0.14% permeation to the basolateral side after 60 minutes. The chlorogenic acid bioavailability detected for golden thistle hydromethanolic extracts was higher than these reported results.

As shown in Figure 5.5e, the trend of chlorogenic acid bioavailability over time was approximately linear. Therefore, apparent permeability (P_{app}) calculation was also possible. The P_{app} was calculated using the formula from Zhou et al. (2015) in Equation 8 below.

$$P_{app} = \frac{\left(\frac{dC}{dt}\right) x\ V}{A\ x\ C_0} \tag{8}$$

in which P_{app} is the apparent permeability of chlorogenic acid in cm/s, dC/dt represents the concentration gradient with respect to time (ppm/min), V is the volume of the basolateral side in mL, A is the insert membrane area in cm^2, and C_0 is the initial concentration of chlorogenic acid applied to the apical side of the insert membrane in ppm. As a result, the P_{app} for the chlorogenic acid in the golden thistle extract mixture was calculated as $(1.82\pm0.07)x10^{-6}$ cm/s. This finding is in accordance with the result reported by Zhou et al. (2015) for *Flos Lonicerae Japonicae*, while slightly higher than that of *Crepidiastrum denticulatum* reported by Lee et al. (2014) for P_{app} values of chlorogenic acid. Mortelé et al. (2021) found 1.3 and 1.4-folds higher P_{app} values than the value calculated for golden thistle in this study.

Chlorogenic acid is an abundant dietary phenolic compound and is found high amounts in coffee. Therefore, *in vitro* and *in vivo* studies have been conducted widely for the chemistry, bioavailability, and potential health effects of that compound. Chlorogenic acid is named after a group of phenolic acids esterified with quinic acid. Generally, caffeoylquinic acid, feruloylquinic acid, and dicaffeoylquinic acid are considered as the group of chlorogenic acids with the highest abundance in 5-*O*-caffeoylquinic acid (Figure 5.6). Chlorogenic acids are easily found in most plants, however, coffee, tea, berry fruits, cocoa, citrus fruits, apples, pears, and globe artichoke are the well-known sources

(Naveed et al. 2018, Stalmach et al. 2011). The compound is the abundant phenolic compound in the families of Asteraceae and Lamiaceae (Naveed et al. 2018) as well.

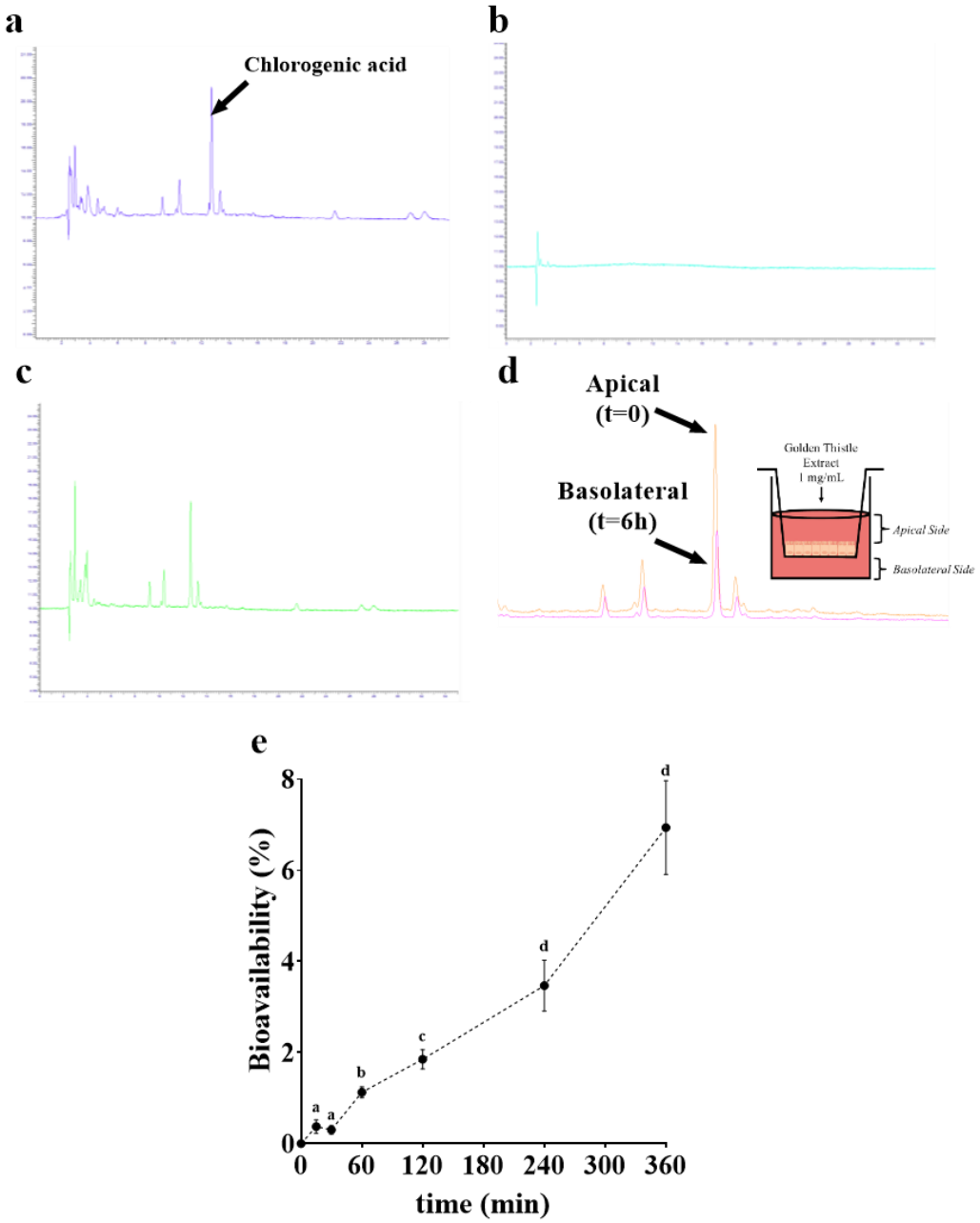

Figure 5.5. *In vitro* Chlorogenic Acid Bioavailability of the Golden Thistle Extract Mixture. Representative chromatograms for (a) extract mixture applied on the apical side, (b) HBSS medium in the basolateral side at the beginning, (c) Bioavailable fraction of golden thistle extract mixture after 6 hours, (d) overlay of the chromatograms from the apical side at the beginning as well as basolateral side at the end of the experiment with a representative experimental scheme, (e) Calculated chlorogenic acid bioavailability in the golden thistle extract mixture with respect to time. In part e, the results are given in mean±SEM for 12 replicates (n=12). Different small-scale letters indicate the statistical difference between the bioavailability percentages for that corresponding timepoint as the results of one-way ANOVA with Tukey's post-hoc test ($p<0.05$). HBSS: Hank's Balanced Salt Solution, ANOVA: Analysis of Variance.

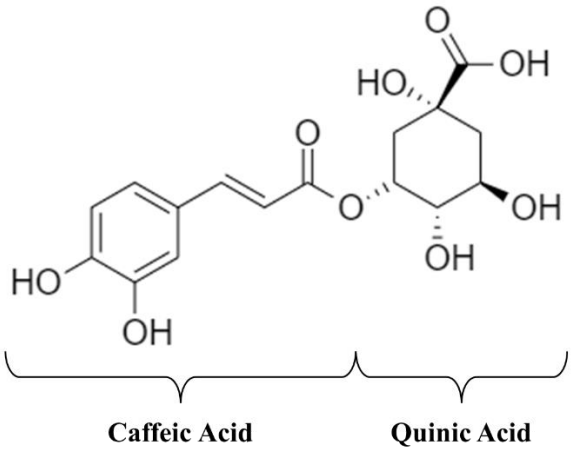

Figure 5.6. Chlorogenic Acid Structure. Chlorogenic acids are a group of phenolic acids esterified with quinic acid. However, 5-*o*-caffeoylquinic acid is the most referred structure. The compound was drawn in ChemDraw software (Perkin Elmer, v.18.1).

Despite the health-promoting effects, chlorogenic acid bioavailabilities have been considered low in literature (Dupas et al. 2006, Mortelé et al. 2021, Kamiloglu et al. 2017, Azuma et al. 2000) *in vitro*. Yet, *in vivo* results might be considered adequate. There are various kinetic absorption models in clinical trials after coffee consumption or other related chlorogenic acid-containing compounds (Del Rio et al. 2010, Olthof, Hollman, and Katan 2001, Stalmach, Williamson, and Crozier 2014). There was also an 8% net absorption for the chlorogenic acid *in situ* perfusion model (Lafay and Gil-Izquierdo 2007). The bioavailable percentages depend on the intake dose (Stalmach, Williamson, and Crozier 2014).

All in all, there are various reports on the passage of chlorogenic acids through the gastrointestinal tract. Some authors suggested no effect by the mammalian enzymes as well as low pH stomach (Dupas et al. 2006, Monente et al. 2015, Stalmach 2012), while some authors reported enzymatic hydrolysis in small amounts (Lafay et al. 2006). Absorption has been reported in ileum and jejunum by (Bohn 2014), while absorption through the stomach was also reported (de Oliveira et al. 2017). Furthermore, microbial esterases further degrade the unavailable chlorogenic acid, and the available phenolic acids can be subjected to another absorption (Naveed et al. 2018).

Much research reported the mechanism of action for chlorogenic acid absorption as paracellular (Farah et al. 2008). Overall, one-third of chlorogenic acid is absorbed before reaching to the microbial activity (Lu et al. 2020). There are some controversial mechanisms suggested by intestinal absorption. Some research revealed that the

absorption was non-saturable passive diffusion, yet active ATP-binding cassette (ABC) efflux transporters were also reported (Stalmach 2012). After absorption, mainly glucuronide, sulfate, and methylated metabolites are formed as the action of phase II enzymes in the liver (Del Rio et al. 2010). Most metabolites are excreted in urine at the last step (Monteiro et al. 2007). There is an overview of chlorogenic acid routes inside the human body as a literature summary in Figure 5.7.

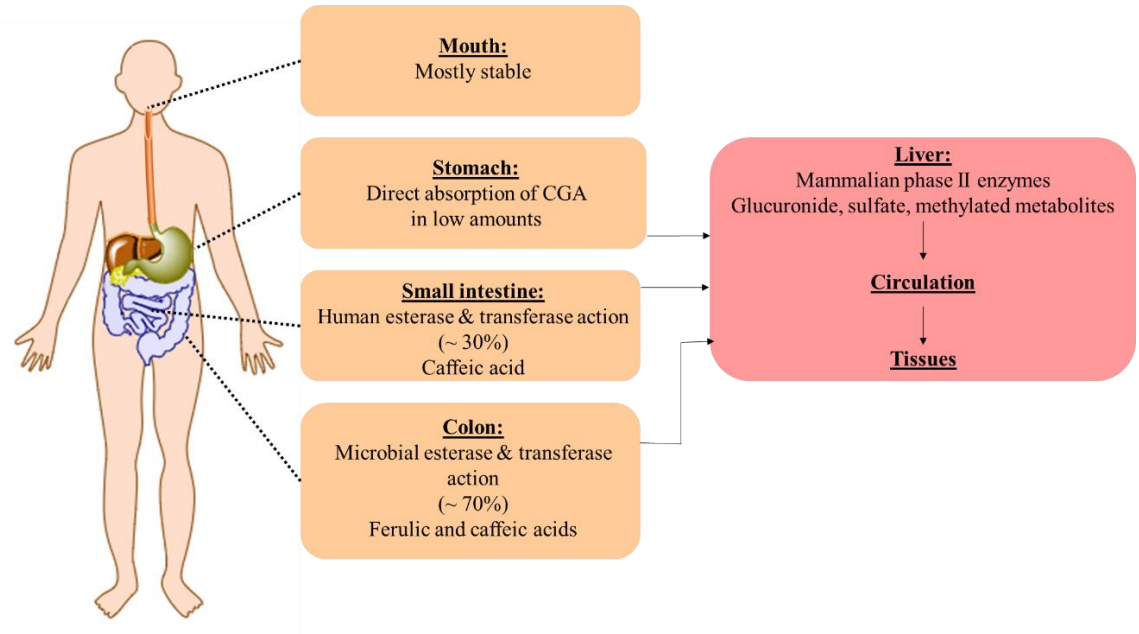

Figure 5.7. The Route of Chlorogenic Acid in the Human Body as an Overview of the Literature.

Among the phenolic acids that constitute the chlorogenic acids, *p*-coumaric and ferulic were stated to be more bioavailable than intact chlorogenic acid (Kishida and Matsumoto 2019). There are four main metabolism possibilities for chlorogenic acid *in vivo*. First, they could be absorbed without transformation. Secondly, there could be small hydrolysis and absorption through the stomach. As the third one, chlorogenic acid would be subjected to gut metabolism with absorption through colon, and lastly, the combination of microbial metabolism as well as mammalian phase II system could take place according to Clifford, Kerimi, and Williamson (2020).

Further research for improving stability and bioavailability might include encapsulation applications. So far, various materials have been used to encapsulate phenolic compounds for enhanced bioavailability as well as stability in the gastrointestinal tract. The encapsulation material provides a physical barrier and controlled release of the compound

of interest using spray drying, freeze-drying or coacervation techniques (Jurić et al., 2020; Marcillo-Parra et al., 2021). Another advantage of encapsulation techniques might be the enhancement of sensory qualities as some of the phytochemical materials are not preferred because of their bitter taste (Delfanian & Sahari, 2020).

Additionally, nanoencapsulation applications are considered for better phytochemical release due to their increased surface area (Esfanjani, Assadpour, & Jafari, 2018). Rashwan et al. (2021), suggests nano-hydrogels or nano-liposomes depending on the solubility properties of the corresponding phytochemical. Therefore, further studies might include nano-hydrogel structures for enhanced chlorogenic acid bioavailability of the golden thistle extract. Yet, *in vivo* trials are still required before commercialization (Reque & Brandelli, 2021).

5.4. Conclusion: *in vitro* Chlorogenic Acid Bioavailability as the Abundant Phenolic Compound in the Golden Thistle

In conclusion, the phenolic profile analysis of golden thistle hydromethanolic extracts showed that chlorogenic acid was the abundant phenolic compound in the samples. When the extract mixture was subjected to an *in vitro* bioavailability study using differentiated CaCo-2 cells, low values in accordance with the literature data was detected. In addition, apparent permeability (P_{app}) calculated for the chlorogenic acid content in golden thistle hydromethanolic extract has been found to be either similar, lower, or higher than some other plants from various families. However, chlorogenic acid has been reported to have health promoting effects even in lower amounts, therefore research for the developments of enhanced chlorogenic acid bioavailability could be studied.

CHAPTER 6

in vitro ANTIDIABETIC EFFECTS OF *Scolymus hispanicus* L. HYDROMETHANOLIC EXTRACT

Some of the well-known Asteraceae plants have been shown to have important antidiabetic activity both *in vitro* and *in vivo*. These antidiabetic activities of the plants are sometimes attributed to the presence of specific phenolic compounds as well as the high abundance of chlorogenic acid in this plant family. Therefore, the possible antidiabetic activity of golden thistle hydromethanolic extract mixture with important chlorogenic acid levels was also evaluated in this section. The effects were investigated in two approaches. In the first one, a starch solution was subjected to a test tube digestion to determine if the extract had any impact on the mammalian glycosidases used in the digestion procedure. However, the results from this section did not cause any important inhibition in the enzymatic activity in the way used for this study. According to the HPLC responses of the test tube digestion, the solely starch solution and the combination of this starch solution with the golden thistle extract mixture released 30.3 and 33.1 mg/mL glucose contents, respectively. There was no significant difference between these two samples ($p>0.05$). In the second approach, the possible effects of the extract in pure glucose absorption were investigated. A similar bicameral insert membrane system used in Chapter 5 with CaCo-2 human enterocytes was utilized for that purpose. The cells were grown and differentiated in these membranes, and the cell integrities were confirmed before the glucose efflux study. Then, one experimental group was pre-treated with the golden thistle hydromethanolic extract for 2 hours, followed by an application of glucose solution at the apical side after discarding the extract. In the second experimental group, the golden thistle extract mixture and glucose solution were introduced to the apical side simultaneously as the co-treatment. All samples, including non-treated control cells, received 25 mM of glucose solution at their apical sides, while all the basolateral sides contained glucose-free Krebs transport buffer. At the end of the assay, the pre-treated group showed 39.4% less glucose absorption than the control group ($p<0.05$), while the co-treated group resulted in 42.6% less glucose ($p<0.05$). Lastly, the mRNA expressions of a common glucose transporter were evaluated with the exact same cells used in the

glucose efflux study. The pre- and co-treated samples resulted in 49 and 66% less GLUT2 levels when compared to the non-treated control cells. The overall results at the functional and molecular levels might indicate that the golden thistle hydromethanolic extract and the phytochemical compounds present in the matrix might be important candidates for antidiabetic activity research for further developments.

6.1. Diabetes Mellitus (DM) and Asteraceae Plants in the Prevention of DM

Diabetes Mellitus (DM) is the severe condition of high blood glucose levels originating from either the absence or impaired action of insulin. DM is considered as one of the leading health problems in the 21st century. According to the latest publication of Diabetes Atlas, 537 million people have been diagnosed with DM, and this number is expected to increase to 783 million in 2045. In addition, the report shared that 6.7 million die due to DM and its complications. Turkey is the leader in DM cases in Europe, with 9 million DM patients in 2021 and an expected 13.4 million by 2045 (IDF 2021).

Blood glucose homeostasis is maintained by a physiologic loop between insulin and glucagon hormones. Briefly, when the blood glucose levels are elevated, beta cells of the pancreas release insulin hormone. Tissues such as skeletal muscle and adipose tissue use the glucose in the bloodstream via GLUT4 insulin-mediated transporter. More importantly, the liver converts the glucose molecules into packed glycogen for storage with tyrosine kinase insulin receptor. As a consequence of these and the usage of glucose within the hours after digestion, the blood glucose levels drop. This time, alpha cells of the pancreas release glucagon hormone for the opposite action. With glucagon, the liver breaks down the glycogen back to glucose molecules to release into the bloodstream (Kaul et al. 2013). An illustrative summary of the blood glucose homeostasis is given in Figure 6.1. However, in DM, this homeostasis cannot be achieved.

There are four main types of DM. The following explanations about the DM types were mostly taken from Diabetes Atlas (IDF 2021) with additions from other resources. Among these types, Type 1 Diabetes Mellitus (T1DM) is an autoimmune disease in which the immune cells of the body attack insulin-producing beta cells of the pancreas. T cells are the most important contributors to T1DM (Ting et al. 2013). In this disease, daily injections of insulin are required to maintain normal blood glucose levels, and factors

such as genetic susceptibility and some viral infections might trigger the occurrence. Three subclasses are formed in T1DM as autoimmune type, idiopathic, and fulminant (Kharroubi and Darwish 2015). The recent type of fulminant T1DM has not only genetic background but is also associated with viral antigens, resulting in rapid and complete beta cell destruction (Shibasaki, Imagawa, and Hanafusa 2013).

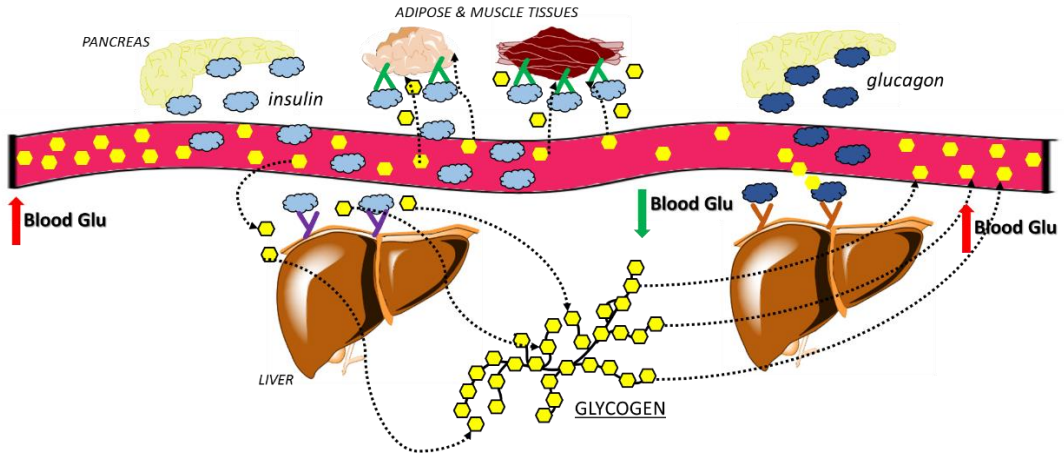

Figure 6.1. Illustrative Representation of Maintaining Blood Glucose Levels. The pancreas releases insulin and glucagon when the blood glucose level is high and low, respectively. The liver is one of the main organs that respond to these hormone releases by either packing glucose molecules into glycogen for storage or breaking down the glycogen into glucose molecules for elevated glucose in the bloodstream. Lastly, adipose and muscle tissues contain insulin-associated glucose transporter of GLUT4, therefore utilizing glucose with insulin action. The visual organ artwork was taken from ChemDraw Software (Perkin Elmer, v.18.1) and combined in MS Office. Glu: Glucose.

On the other hand, in Type 2 Diabetes Mellitus (T2DM), although insulin is produced normally, the cells resist to the insulin action, and thus the glucose cannot get into the cell, resulting in high blood glucose freely. T2DM constitutes 90% of all DM cases. Factors like urban lifestyle, overweight, age, family history, inadequate diet, and physical activity are strongly linked to T2DM (Ginter and Simko 2013). Especially, obesity is considered with an expectation of T2DM (Al-Goblan, Al-Alfi, and Khan 2014, Ginter and Simko 2013). Sometimes, T2DM can be asymptomatic in the early stages, making it difficult to detect and diagnose (Kharroubi and Darwish 2015).

Another type, Gestational Diabetes Mellitus (GDM), occurs during pregnancy and may be recovered after birth. Mothers prone to being overweight during pregnancy are more likely to develop GDM. This condition occurs approximately in 7% of all pregnancies with hypertension, pre-eclampsia, retinopathy, and risk of future diabetes for mothers

(Imam 2013). There are serious consequences of GDM, most commonly macrosomia, which may require cesarean section (Imam 2013). In addition, newborns might suffer from T2DM if their mother had GDM in pregnancy. Long-term complications such as obesity might also be expected in GDM (McIntyre et al. 2019).

In rare cases, some other types of DM might be diagnosed. Examples of these types are monogenic diabetes, or other DMs resulting from the impaired pancreas, endocrine disorders, drugs that cause complications of insulin secretions, or other genetic syndromes associated with DM. For instance, maturity-onset diabetes of the young (MODY) is associated with six genes (Maraschin 2013).

For most of these DM types, increased levels of pro-inflammatory cytokines are detected in the bloodstream (Lontchi-Yimagou et al. 2013, Bascones-Martínez et al. 2013). In addition, some macro- and microvascular complications, immune system impairment, and periodontal or foot diseases have been associated with various DM types (Kaul et al. 2013). Macrovascular complications can be listed as coronary heart diseases, ulceration, gangrene, and amputations, while microvascular complications are neuropathy, nephropathy, and retinopathy (Kaul et al. 2013).

Nutritional recommendations have been proposed for balanced blood glucose levels. Recently, various plant phytochemicals have been reported to affect DM and blood glucose levels via alterations in glucose metabolism, hypolipidemic effects, pancreatic effects, antioxidative effects, curative effects on diabetes complications, and insulin-like effects (Chan, Ngoh, and Yusoff 2012). Extracts, isolated compounds, or developed formulations are tested regarding antidiabetic activity *in vivo* or *in vitro*. Sometimes, the inhibition capacity of a specific mammalian glycosidase can also be attributed to antidiabetic activity.

Asteraceae plants are widely studied species for their antidiabetic effects. For instance, the Turkish endemic plant *Achillea cucullate* showed significant α-glucosidase inhibition capacity (Eruygur et al. 2019). In addition, *Argyrantheum pinnatifidum*, *Helichrysum melaleucum*, and *Phagnalon lowei* inhibited both amylase and glucosidase enzymes (Spínola and Castilho 2017). Furthermore, *Helichrysum plicatum* spp. *plicatum*, which is used for treating the complications of DM in folkloric medicine, has been proved for this effect in streptozotocin-induced diabetic rats (Aslan et al. 2007). *Tanacetum nubigenum* (Khan et al. 2018), *Achillea millefolium* (Chávez-Silva et al. 2018), *Pallenis spinosa* (Khettaf and Dridi 2022), *Centratherum anthelminticum* seeds (Arya et al. 2012) are some other examples of ameliorating DM markers in diabetic mice or rat models *in vivo*.

Golden thistle plant has been reported for antidiabetic activity by different researchers. Marmouzi et al. (2017) reported α-amylase and α-glucosidase inhibitions, while Berdja et al. (2021) reported an improvement in glucose tolerance and reduced lipid accumulation in rats with a hyperfatty diet. Therefore, this chapter aimed to investigate the antidiabetic activity of golden thistle hydromethanolic extract mixture in two *in vitro* models using test tube digestion and CaCo-2 cell line.

6.2. Materials and Methods

Two different approaches were conducted for *in vitro* antidiabetic activity determination. First, starch digestibility was evaluated to test whether the golden thistle hydromethanolic extract affected digestive enzymes. In the second approach, a glucose efflux test was applied using differentiated CaCo-2 cells on the bicameral insert membrane system. Further mRNA expression of the cells used in the glucose efflux model was also determined.

6.2.1. Materials and Chemicals Used

1-(4,5-Dimetylthiazol-2-yl)-3,5-diphenylformazan (MTT, M2003), pepsin (P7000), guar (G4129), pancreatin (P7545), amyloglucosidase (A7095), invertase (I4504), Minimum Essential Medium (MEM, M4655), non-essential amino acid solution (M7145), sodium pyruvate (S8636), penicillin-streptomycin solution (P4333), and Trizol® Reagent (T9424) were purchased from Sigma (Mannheim, Germany). Fetal bovine serum (FBS, 10270) was from Gibco (Thermo Fisher Scientific, Massachusetts, USA), and 12-well insert membranes for the glucose efflux studies (Transwell®, 3460) were of Corning (New York, USA). cDNA synthesis kit (EQ003), SYBR-Green mix (EQ001), and primers of mRNA expression studies (Invitrogen) were purchased from ELK Biotechnology Co. Ltd. (Wuhan, China). All other chemicals were analytical grade and from either Sigma or Merck. MilliQ water was used when needed in the experiments.
Similar to the *in vitro* chlorogenic acid bioavailability study in Chapter 5, throughout this part, a mixture sample was prepared by using three individual hydromethanolic extracts

in equal amounts and utilized as the main sample of the experiments at 1 mg/mL concentration. Unless stated otherwise, all water stated in the experiments was MilliQ®.

6.2.2. *in vitro* Digestion Procedure

Starch digestibility was tested within an experimental design of 4 samples. A GTE sample in which no enzyme was used in the digestion procedure (GTE_{blank}), a solely digested GTE, control group of 1% starch solution (Starch), and 1% starch solution to contain 1 mg/mL GTE (Starch+GTE) at the end of the digestion procedure. *In vitro* digestion was a modified procedure of Englyst, Hudson, and Englyst (2000), which was published earlier (Ozel-Tasci et al. 2020). Before applying the procedure, a gastric solution containing 1% pepsin and 1% guar gum in 0.05 M HCl was prepared. Also, an intestinal solution was prepared. For that purpose, 3 g pancreatin was mixed with 20 mL of water and vortexed for about 10 minutes. Then, the mixture was centrifuged at 6800xg for 10 minutes, and 15 mL of the supernatant was taken. 0.666 mL amyloglucosidase and 1 mL of 10 mg/mL invertase were added into this supernatant to achieve the final intestinal solution. During the *in vitro* digestion procedure, the samples were mixed with 5 mL of gastric solution. This gastric mixture was incubated at 37°C and 100 rpm for 30 minutes. In addition, glass balls were added to each sample tube to mimic the peristaltic movement of the gastrointestinal tract. At the end of the gastric phase, 5 mL of 0.25 M sodium acetate solution was added to neutralize the pH, and 2.5 mL of intestinal solution was added. The intestinal phase took place under the same conditions for 2 hours. The test tubes were boiled for 30 minutes to terminate the digestive enzymes. At the end of this application, released glucose was tested with high pressure liquid chromatography (HPLC).

6.2.3. Cell Maintaining and Cytotoxicity

A similar application to *in vitro* chlorogenic acid bioavailability test was designed for the glucose efflux study. CaCo-2 cells were grown in T-75 flasks with MEM containing 15% FBS, 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin-streptomycin solutions. All maintaining, differentiation and experimental periods took

place in a 37°C incubator with 5% CO_2. The passage numbers were kept between 30-40 throughout the experiments.

1 mg/mL concentration that was shown to be non-toxic to the CaCo-2 cells was also utilized throughout the glucose efflux study for longer periods (Fig. 5.4a). Therefore, no additional cytotoxicity test was conducted for this part of the study.

6.2.4. Glucose Efflux Model

CaCo-2 cells were seeded and incubated until full confluency in 12-well bicameral insert membrane system for about 2-3 days. Then, 21 days of the post-confluency period was started with fresh medium changes every other day. TEER measurements were done to test the cell integrity before the glucose efflux assay. In addition, a phenol red assay was conducted with the efflux study. The apical parts of the efflux study contained 30 mM phenol red solution. At the end of the experimental period, the phenol red portion passed through the basolateral part was evaluated by measuring the absorbances at 560 nm. The absorbance of apical 30 mM phenol red concentration in Krebs transport buffer was assumed to be 100%, and the absorbance of the end-basolateral sample was normalized according to this value. For the glucose efflux study, there were 3 experimental groups which all given 25 mM of glucose solution in Krebs transport buffer at the apical side. In the first experimental group of non-treated control cells, only 25 mM glucose was given to the apical side. The second experimental group was pre-treated with 1 mg/mL of golden thistle extract mixture (GTE) for 2 hours. At the end of the period, extract containing solution was discarded, and 25 mM of glucose solution was applied to the apical side. The sample was named as pre-treated group. Lastly, both 1 mg/mL GTE and 25 mM of glucose solution were introduced to the apical side simultaneously, and this sample was named as co-treated group. In all samples, basolateral sides contained only glucose-free Krebs transport buffer. Periodical samples were taken from the basolateral sides and analyzed on HPLC for their glucose contents (Fig. 6.2).

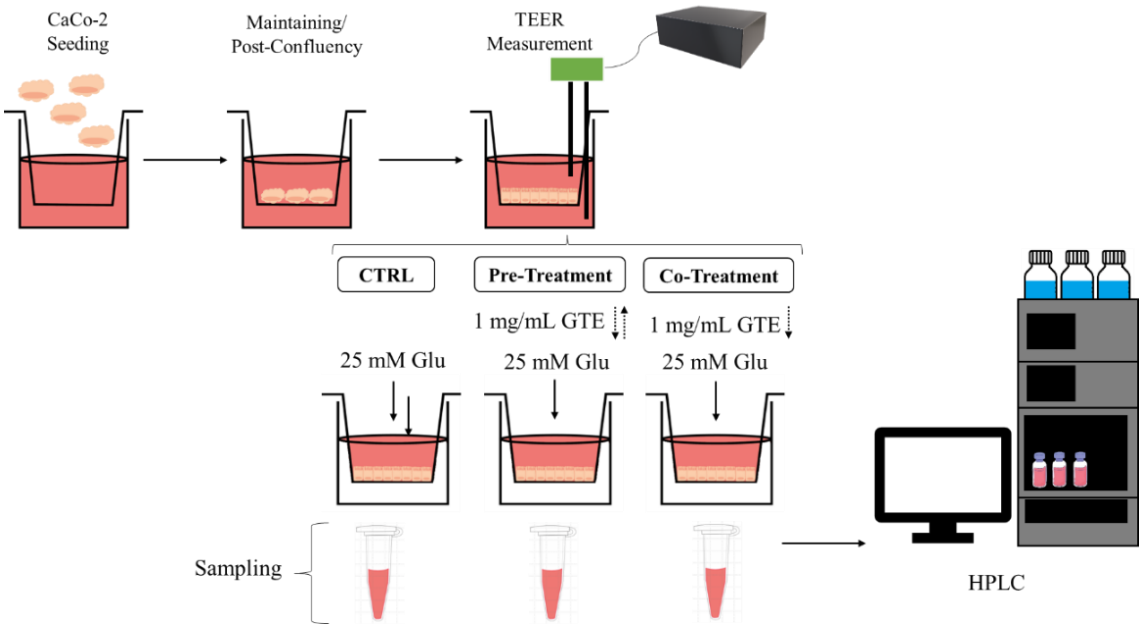

Figure 6.2. Summary of Glucose Efflux Model Proposed. 1 mg/mL GTE was either pre- or co-treated with differentiated CaCo-2 human enterocyte cells at the apical side; samples were taken periodically at the basolateral side and analyzed on HPLC. TEER: Transepithelial Electrical Resistance, GTE: Golden Thistle Extract, Glu: Glucose, HPLC: High Pressure Liquid Chromatography

6.2.5. High Pressure Liquid Chromatography (HPLC) Conditions

In the starch digestibility and glucose efflux study, the glucose content was determined using HPLC (Perkin Elmer, Waltham, MA) with the method from Surek and Buyukkileci (2017). Aminex-87H (Bio-Rad, Hercules, CA) column was used at 80°C. An isocratic elution of 0.6 mL/min flow rate was applied with 5 mM H_2SO_4 as the mobile phase. The glucose response was measured using a refractive index (RI) detector.

6.2.6. RT-qPCR

Changes in the mRNA expressions of the common glucose transporter GLUT2 was also determined using the cells that are used in the glucose efflux model. The overall procedure consisted of three main steps: total RNA isolation, cDNA synthesis, and RT-qPCR. In the first step, the cells were lysed with 1 mL Trizol® reagent for each membrane, followed by an incubation of 5 minutes at room temperature and centrifugation at 12000xg for 5 minutes sequentially. Then, the supernatants were taken into new centrifuge tubes, 0.2

mL of chloroform was added to each tube, and the tubes were shaken vigorously and incubated at room temperature for 3 minutes. For phase separation, the tubes were centrifuged at 12000xg for another 10 min., and the aqueous phase was taken into new microcentrifuge tubes. 0.5 mL of isopropyl alcohol was added into each tube and centrifuged again at 12000xg for 8 min. to precipitate the RNAs. The pellets were further washed with 75% ethanol with centrifugation at 7500xg for 3 minutes for an overall 2 times. At the end of this step, the ethanol residues were discarded, and the pellets were dried on ice with the caps opened for 5 minutes. In the last step of total RNA isolation, the pellets were dissolved in DEPC water. Before cDNA synthesis, the concentrations of the dissolved isolates were measured in μDrop at 240 and 280 nm (ThermoskanGO, Thermo Scientific, Massachusetts, USA).

From these RNA isolates, complementary DNAs were prepared using a PCR synthesis kit (ELK Biotech, Wuhan, China) for each 2 μg of RNA according to the kit instructions. For this synthesis, first, the genomic DNA was removed in a mixture of 2 μL gDNA eraser buffer, 1 μL gDNA eraser, 1 μg of template RNA, and RNAse free water to a final volume of 10 μL. The mixture was incubated at room temperature for 5 minutes. Next, 2 μL of RT primer mix, 1 μL of dNTPs (10 mM), and RNAse-free water to a final volume of 15 μL. This second mixture was incubated at 70°C for 5 minutes and transferred onto the ice to cool. Then, 4 μL RT buffer and 1 μL RTase was added, incubated at first 37°C for 60 minutes, then at 95°C for 5 minutes, and cooled.

Lastly, RT-qPCR conditions were constituted using 10 μL of SYBR dye, 0.4 μL forward and 0.4 μL reverse primers (5 μM each), 2 μL of synthesized cDNA, 0.4 μL ROX dye, and 6.8 μL of water. Human CyclophilinA was used as the housekeeping gene for that procedure, and the samples were subjected to an RT-qPCR procedure (ABI StepOne Plus, Lifetech, CA, USA) as follows: 95°C/10 min., 40 cycles (95°C/10s, 60°C/1 min.) and 95°C/15s, 60°C/1 min. steps. The data obtained were analyzed with the $2^{-\Delta\Delta Ct}$ method, and the results were given as fold changes. The primers used for housekeeping cyclophilin A (CypA) and glucose transporter (GLUT2) are given in Table 6.1.

6.2.7. Statistical Analyses

All data were given as mean±standard error for at least three experiments ($n \geq 3$). The samples were compared using either 2 sample t-tests or one-way analysis of variance

(ANOVA), depending on the samples. For ANOVA, Tukey's post hoc test was used for result comparisons ($p<0.05$).

Table 6.1. Primer sequences used in the RT-qPCR for the determination of mRNA levels.

Primer	Forward (3'-5')	Reverse (5'-3')	PMID/Reference
CypA	TACGGGTCCTGGCATCTTG	CGAGTTGTCCACAGTCAGCA	(Boztepe and Gulec 2018)
GLUT2	CTCTCCTTGCTCCTCCTCCT	TTGGGAGTCCTGTCAATTCC	25010715

6.3. Results and Discussion

For the antidiabetic activity studies, first, the effects on the digestive enzymes were investigated by applying a test tube digestion method. Then, a glucose efflux assay was conducted to understand if the golden thistle extract mixture had any effects solely on glucose absorption. In addition, the same cells used in the glucose efflux study were subjected to an mRNA expression analysis for the possible mechanism of action.

6.3.1. Effects on Starch Digestibility

In the first part, the effects on starch digestibility were evaluated (Fig. 6.3). For that purpose, the golden thistle extract mixtures included in that assay were 12 mg in each extract, including sample as the final volume of the test tube digestion procedure is 12 mL, the concentration would become the same as in the rest of the studies. In the first sample, the extract sample was subjected to a test tube digestion without using any enzymes. However, other parameters such as shaking, temperature, and presence of guar, HCl, and sodium bicarbonate were present. This sample was named as GTE_{blank}. The results showed 0.04 mg/mL glucose release from the extract with the influence of chemical or physical attributes such as pH or mimicking peristaltic movements with the glass balls. When the extract mixture sample was digested using the mammalian glycosidases, 1.61 mg/mL glucose was released at the end. Although the glucose released from the digested extract was higher than the nondigested sample, there was not any

significant difference between these two samples when the starch solution was not included ($p>0.05$).

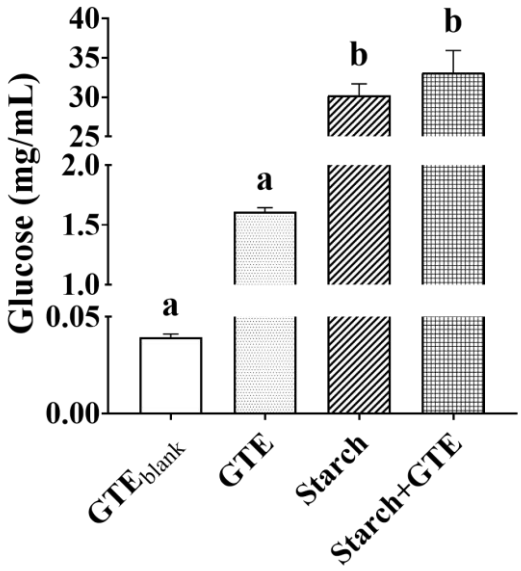

Figure 6.3. Effects of the Golden Thistle Hydromethanolic Extract on Starch Digestibility. A test tube digestion procedure was applied to GTE, Starch, and Starch+GTE samples, including all steps of carbohydrate digestion. However, the GTEblank sample was subjected to the same experimental conditions, excluding the digestive enzymes. The results are given as mean±SEM for three replicates (n=3). Different letters above the bars indicate the statistical significance of the results of one-way ANOVA with Tukey's post-hoc tests ($p<0.05$). GTE: Golden Thistle Extract.

As the control of the assay, 1% starch solution was digested, and 30.25 mg/mL glucose was determined. The glucose release from the starch sample was approximately 750 and 19-folds higher than the GTE_{blank} and GTE sample glucose releases, respectively ($p<0.05$). However, when the extract mixture was added to the starch solution before digestion, no effects on the glucose release could be detected. The final glucose release from the combination of starch and extract mixture was 33.13 mg/mL. There were no differences between the starch solution containing extract and the control ($p>0.05$). Regarding these results, it can be said that the golden thistle hydromethanolic extract mixture did not show any inhibitory effects in the model used for the starch digestion for this part. However, an absolute ethanol extract of the golden thistle has been reported to be effective in both amylase and amyloglucosidase action (Marmouzi et al. 2017). Yet, as the experimental procedures are entirely different, it would be speculative to compare the current results obtained from the complex and viscous test tube digestion procedure to the enzymatic activity-based assays.

6.3.2. Effects on Glucose Efflux

Figure 6.4 shows the glucose efflux study using the bicameral insert membrane system with CaCo-2 cells. The increasing trend in glucose absorption with respect to time can be seen in 120 minutes of the experimental period (Fig 6.4a). In the first 30 min., there were no considerable differences between the samples as the non-treated control, pre-treated, and co-treated samples resulted in 0.039, 0.028, and 0.015 mg/L glucose contents, respectively. The same could be said for the first hour, either. The control sample had 0.083, the pre-treated sample had 0.076, and the co-treated sample had 0.056 mg/L glucose contents at their basolateral sides with no significant differences between the samples ($p>0.05$). However, the differences between the non-treated control and the extract-treated samples became apparent after 90 minutes. There were 0.139, 0.010, and 0.093 mg/L glucose in the basolateral sides of control, pre-, and co-treated samples, respectively ($p<0.05$). The pre-treated sample had 28.1%, while the co-treated sample had 33.1% less glucose after 90 minutes (Fig. 6.4b). Similarly, at the end of the experiment, the pre-treated sample resulted in 39.4%, and co-treated sample resulted in 42.6% less glucose with the values of 0.089, 0.094, and 0.155 mg/L glucose contents for co-treated, pre-treated, and non-treated control samples, respectively ($p<0.05$).

The same difference was observed when the area under curve (AUC) values were calculated for the samples of glucose efflux (Fig. 6.4c). The non-treated control sample was calculated to be 9.35, while pre-treated and co-treated samples resulted in 6.99 and 5.37 AUC values, respectively. However, in the AUC values, only the co-treated sample resulted in significantly different AUC compared to the non-treated control sample ($p<0.05$). The co-treated sample was 49% lower than the control for the area under curve.

In addition to the TEER measurements of cell integrity, a phenol red assay was also applied during the glucose efflux studies by introducing phenol red solution at the apical side and checking the transferred phenol red content at the basolateral side at the end of the experiment. None of the samples used in the data exceeded 5% of phenol red transfer, indicating the cell integrity as an alternative method (data not shown).

As the last assay of *in vitro* antidiabetic activity, relative mRNA expression of a common glucose transporter of the same cells used in the glucose efflux study was utilized to get an insight into the changes at the molecular level (Fig. 6.5). The results showed that the golden thistle extract applications significantly downregulated GLUT2 mRNA

expressions in CaCo-2 enterocytes. Both pre- and co-treatments were significantly lower in GLUT2 mRNA expressions with 49% and 66% reductions, respectively ($p<0.01$).

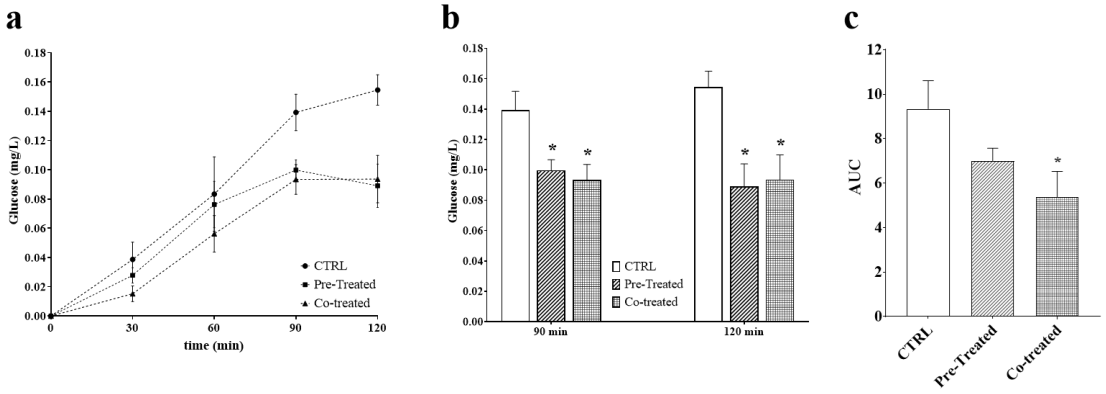

Figure 6.4. Effects of the Golden Thistle Extract on Glucose Efflux of CaCo-2 Cells for Different Treatments. CaCo-2 cells grown and differentiated on the bicameral insert membrane system were either pre-treated with the golden thistle extract for 2 hours or treated simultaneously with the glucose application (co-treated). All samples, including the non-treated control group, was given 25 mM of glucose solution in Krebs transport buffer at the apical side at t=0. Periodical sampling and detection of glucose content in HPLC resulted in (a) glucose contents at the basolateral side with respect to time, (b) representation of the final glucose contents of the samples at the basolateral side after 90 and 120 minutes, and (c) the AUC values calculated for glucose efflux data in (a). The results are given in mean±SEM for 3 replicates. Statistical analyses were conducted by two-sample t-tests comparing the non-treated control sample with either pre- or co-treated samples (*$p<0.05$). CTRL: Control, AUC: Area Under Curve.

Various plants have been shown to change carbohydrate digestion and/or glucose absorption in literature (Nistor Baldea et al. 2010). Although in literature, glucose uptake assays are more frequently used instead of glucose efflux studies. Yet, there are also glucose efflux studies with significant results utilizing different types of tea samples in similar conditions to this current study (Liu et al. 2021). The researchers reported that glucose absorption decreased with increasing concentrations in their work. They also reported changes in SGLT1 and GLUT2 mRNA expressions with the applications of black, oolong, or green teas to the CaCo-2 cells. GLUT2 is more widely studied among the glucose transporters in literature when compared to the other transporters, such as SGLT1, both in mRNA and protein levels (Alzaid et al. 2013, Manzano and Williamson 2010).

In this part, the effects shown in the antidiabetic activity assays *in vitro* might be attributed to the abundant presence of chlorogenic acid, which has been reported in Chapter 5. However, to confirm the contribution of the chlorogenic acid to the antidiabetic activity of the golden thistle hydrotmethanolic extract, an experimental design including pure

chlorogenic acid as a positive control sample must be conducted. Still, as the chlorogenic acid is a very well-known antidiabetic compound both *in vitro* and *in vivo* (Singh et al. 2021, Ong, Hsu, and Tan 2013), the possibility of the chlorogenic acid present in the golden thistle might be commented when regarding the results obtained from this part of the study.

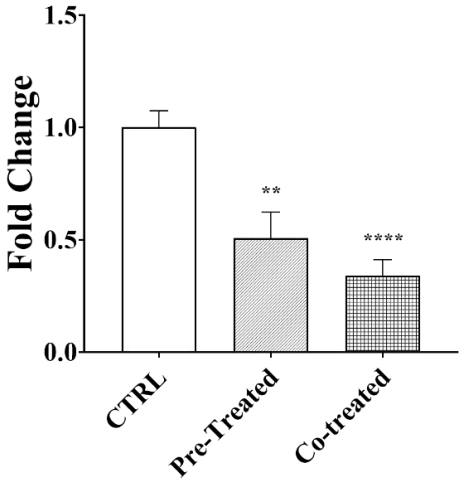

Figure 6.5. Relative GLUT2 mRNA Expressions of the Golden Thistle Extract-treated CaCo-2 Cells of the Efflux Study. The cells used in the glucose efflux study were subjected to total RNA isolation, cDNA sysntesis, and RT-qPCR steps for GLUT2. The results are given as mean±SEM for at least 3 replicates (n≥3). One-way ANOVA with Tukey's post-hoc test was utilized to analyze the statistical significance. **p<0.01, ****p<0.0001. mRNA: messenger ribonucleic acid, CTRL: control, cDNA: complementary deoxyribonucleic acid, RT-qPCR: Real-time quantitative polymerase chain reaction, GLUT2: Glucose transporter 2, ANOVA: Analysis of Variance.

6.4. Conclusion: Golden Thistle Extract Mixture Reduced Glucose Efflux via Downregulating GLUT2 Relative mRNA Expressions in CaCo-2 Cells

The overall antidiabetic activity of the golden thistle hydromethanolic extract might be concluded in two steps. First, the possible effect on the glycosidases of the conditions mimicking the physiological conditions of the human digestive system was tried; however, no inhibitory activity of the phytochemicals present in the extract could be found from this approach. In the second attempt, a glucose efflux study was applied to evaluate the effects of the phytochemical constituents of the extract on pure glucose absorption of CaCo-2 human enterocytes. The extracts were either pre-treated or co-treated with the glucose solution. From this section, the co-treated extract showed better

decreases in the overall glucose efflux of the CaCo-2 cells. For further insight, the cells used in the efflux study were subjected to an analysis of mRNA expressions, and it was found that both pre- and co-treated samples resulted in lower GLUT2 mRNA expressions. Therefore, it can be concluded that the golden thistle hydromethanolic extract showed promising results in terms of antidiabetic activity *in vitro*, making itself a candidate for further *in vivo* studies with identifications of effective molecules of molecular groups.

CHAPTER 7

EFFECTS OF *Scolymus hispanicus* L. HYDROMETHANOLIC EXTRACT IN SYSTEMIC AND COLONIC MODELS OF INFLAMMATION *in vitro*

Inflammation can be linked to various diseases both in systemic and local pathologic conditions. Although inflammation is required for the defense system in the body, overproduction of the markers can cause additional problems. Therefore, a balance in the inflammatory markers is needed. Plants and plant-based molecules are therefore widely studied for their effects on inflammatory conditions. One of the important plant families in this context can be suggested as the family of Asteraceae. Different species of this family have been reported as preventive and/or therapeutic agents in inflammation. As the golden thistle is an Asteraceae plant, this part aimed to evaluate the preventive effects of the hydromethanolic extract obtained from golden thistle in two models of inflammation *in vitro*. In the first model, systemic inflammation was applied by using RAW 264.7 murine macrophage cells. The cells were pre-treated with different concentrations of the extract mixture as 50, 150, and 500 µg/mL for 2 h, and then 0.5 µg/mL lipopolysaccharide (LPS) was introduced to the cells to induce the inflammation. Dose-dependent decreases were detected in all markers determined. 150 and 500 µg/mL pre-treatments resulted in 31 and 47% lower nitric oxide releases, respectively ($p<0.05$). In addition, 31.8 and 45.1% lower tumor necrosis factor-alpha (TNF-α) were observed in the same treatment concentrations. On the other hand, all concentrations of pre-treatments showed significantly lower values of IL-6 in 15.9 and 36.2% lower range ($p<0.0001$). Inflammation also affects the health and well-being of local parts of the body. The gastrointestinal tract is one of the most important examples of the body as it faces foreign compounds and microorganisms after ingestion. Therefore, as it is prone to local inflammation, a colonic inflammation model was chosen as the local inflammation model in the study. For that, LPS was given to the RAW 264.7 cell line for 12 h to obtain an inflammatory medium (IM), which was used as the inflammation inducer of the model. CaCo-2 human enterocyte cells were pre-treated with the same concentrations of the

golden thistle hydromethanolic extracts for 4 h, and the IM was given for local inflammation for an additional 24 h. Although the reductions in IL-6 and IL-8 cytokine releases were not dose-dependent, all concentrations showed significant reductions for both cytokines. IL-6 reductions were in a range of 15.6-19.5%, whereas IL-8 reductions were in a range of 12.6-17.4% in increasing concentrations ($p<0.01$). In conclusion, the golden thistle hydomethanolic extract mixture resulted in lower pro-inflammatory cytokine releases and other tested inflammation markers in both systemic and colonic models of inflammation. In further studies, the biologically active molecules or molecular groups might be determined and used as candidate molecules for *in vivo* trials.

7.1. Inflammation, Anti-inflammatory Plants, and Asteraceae Family Against Inflammation

Inflammation is the response of the body to any abnormal situation. In other words, inflammation can be defined as the reaction to infections, irritations, or injuries (Stankov 2012). It can be considered the starting point for most diseases due to its complex occurrence (Scott et al. 2004). Clark (2007) and Sompayrac (2012) summarize the first innate immune system defense in the following sentences. In the case of inflammation or injury, the first cell type that responds immediately is the macrophages. This first defense of the immune system constitutes the beginning of innate immunity. The macrophages are not only able to attack and eliminate the invading microorganisms but are also capable of releasing pro-inflammatory cytokines. These cells are found with dendritic cells in the normal state, relatively inactive around the vulnerable parts for infections, for example, epithelium. This phenomenon initially provides an active, non-specific, and fast defense. Cytokines can be defined as hormone-like protein structures that are released during an inflammatory process to mark the inflammatory area and seek for other immune cells for help. In the progressing steps of inflammation, other immune cells such as B cells, helper and killer T cells, and natural killer (NK) cells also take place. Together, these immune cells can eliminate the infection by their specific mechanisms and provide homeostasis in the body.

Being the messengers of the inflammatory site, cytokine release plays an essential role in the inflammatory response. Normally, the body needs inflammation to maintain health and well-being. However, in some cases, overproduction of inflammatory cytokines may

lead to other related diseases. There are some non-specific cytokines that can be considered as the early messengers of the inflammatory response. Various inflammatory diseases occur due to overproduced cytokines and other compounds such as nitric oxide (NO) (Sharma, Al-Omran, and Parvathy 2007, Tak and Firestein 2001).

Inflammation can occur in the bloodstream and affect various tissues, known as systemic inflammation. In this type of inflammation, multiple tissues may be affected. To name a few, inflammation-associated diseases could be listed as asthma, rheumatoid arthritis, lupus, psoriasis, spondyloarthritis, obesity, cardiovascular diseases (CVD), neurodegenerative diseases, and cancer (Barnes and Karin 1997, Okin and Medzhitov 2012, Steyers and Miller 2014).

On the other hand, local inflammation can take place in different parts of the body and can be associated with related diseases. As the gastrointestinal system and the gastrointestinal tract are highly vulnerable parts that can be infected in the body, colonic inflammation is one of the best examples of local inflammations. Colonic inflammation affects the gastrointestinal tract and leads to the formation of inflammatory bowel diseases (IBD), namely Chron's disease and ulcerative colitis (Barnes and Karin 1997). At first, the *hygiene hypothesis* was proposed for the incidence of autoimmune diseases as the result of improved personal hygiene leading to the attack of the immune system on the body itself. However, recent findings also suggested the impact of the Western diet on immunological disorders (Thorburn, Macia, and Mackay 2014). This suggestion also confirms the importance of the gastrointestinal tract in inflammatory conditions for local colonics and related systemic inflammation.

Plant-based remedies or other formulations have long been utilized in local or traditional medicine to reduce inflammatory responses. In particular, plants from the Asteraceae family have been widely used as anti-inflammatory agents both for preventive and treatment purposes (Panda et al. 2019). Among them yarrow (*Achillea millefolium* L.), artichoke (*Cynara scolymus* L.), chicory (*Cichorium intybus* L.), and dandelion (*Taraxacum* spp.) are some of the well-known anti-inflammatory plants from Asteraceae (Rolnik and Olas 2021, Tadić et al. 2017). In the study of Bessada, Barreira, and Oliveira (2015), there is a comprehensive review of the anti-inflammatory activities of the family. As a member of the Asteraceae, the golden thistle plant might have some preventive effects in reducing the inflammatory response. Therefore, this part aimed to evaluate the preventive effect of the golden thistle hydromethanolic extract mixture on systemic and colonic models of inflammation *in vitro*. To achieve this, RAW 264.7 mouse

macrophages, as well as CaCo-2 human adenocarcinoma cell line, were used in the experimental models. Some well-known markers of the inflammation studied were monitored throughout this part.

7.2. Materials and Methods

This part explains the constitution of *in vitro* systemic and colonic inflammation models using RAW 264.7 and CaCo-2 cell lines and the methodologies used to determine inflammatory markers. Throughout the assays, the negative control was the cells treated with 3% FBS containing RPMI medium, while the positive control was the mediums treated with 0.5 μg/mL of lipopolysaccharide (LPS). 50, 150, and 500 μg/mL extract mixtures were used as the experimental samples for the assays.

7.2.1. Materials and Chemicals Used

Phosphate buffered saline (PBS) tablets (P4417), Minimum Essential Medium (MEM, M4655), non-essential amino acid solution (M7145), sodium pyruvate (S8636), RPMI-1640 Medium (R8758), penicillin-streptomycin solution (P4333), trypsin-EDTA solution (T4049), 1-(4,5-Dimetylthiazol-2-yl)-3,5-diphenylformazan (MTT, M2003), lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (L2630), sulfanilamide (S9251), and N-(1-Naphthyl)ethylenediamine dihydrochloride (NED) (33461) were purchased from Sigma (Mannheim, Germany). Dimethyl sulfoxide (DMSO, 102952) was from Merck (Darmstadt, Germany), fetal bovine serum (FBS) (10270), and heat inactivated FBS (10500) were from Gibco (Thermo Fisher Scientific, Massachusetts, USA). Sodium nitrite (969.123) and orto-phosphoric acid (85%) (959.066) were from Isolab Chemicals (Eschau, Germany). Mouse TNF-α (EK0527), mouse IL-6 (EK0411), human IL-6 (EK0410), and human IL-8 (EK0413) enzyme-linked immunosorbent assay (ELISA) kits were obtained from Boster Biological (California, US).

Similar to the bioavailability study in Chapters 5 and 6, the mixture sample was utilized as the experimental sample in equal volume from each extract in varying concentrations. Unless stated otherwise, all water stated in the experiments was MilliQ®.

7.2.2. Cell Cultures and Maintaining

RAW 264.7 murine macrophages were used in both inflammation models. They were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS. On the other hand, CaCo-2 cells were utilized for the colonic inflammation model. They were maintained in MEM containing 15% FBS, 1% non-essential amino acid, 1% sodium pyruvate, and 1% penicillin-streptomycin solutions.

All maintaining, differentiation, and experimental periods took place in a 37°C incubator with 5% CO_2. The passage numbers were kept between 30-40 throughout the experiments for CaCo-2 cells, while the passage number did not exceed 28 for RAW 264.7 macrophages.

7.2.3. Cytotoxicity

Cytotoxicity assay was used to confirm cell viability in the corresponding concentration and period of the applied inflammation model. The detailed cytotoxicity determination was also given in Section 5.2.3. However, the concentrations and periods were slightly different. 50 and 500 µg/mL extract mixture concentrations were applied as the experimental treatments to both cell lines as the lowest and the highest concentrations of the inflammation models. The incubation period was chosen as 4 hours as it was the maximum period for the colonic inflammation model. The shorter periods were assumed to be viable after confirming the viability after 4 hours.

7.2.4. Systemic Inflammation Model

As stated earlier, only RAW 264.7 murine macrophages were used in the systemic inflammation model. For the application, the cells were seeded in $1x10^6$ cell/well density in 12-well cell culture plates and waited overnight for attachment. The next day, a starving step was applied to eliminate the effects of FBS components on the inflammatory condition. For that purpose, the cell culture medium was changed with 3% FBS containing RPMI starving medium for 6 hours. Then extract mixture was introduced to

the cells at 50, 150, and 500 µg/mL concentrations in 3% FBS containing RPMI medium as pre-treatments for 2 hours. After 2 hours, the extract containing cell culture mediums were discarded, and 0.5 µg/mL LPS containing new medium was added into each well for the formation of inflammation for an additional 12 h. At the end of the period, samples were taken from the mediums and evaluated for mouse TNF-α and IL-6 with ELISA. The experimental design is given in Figure 7.1.

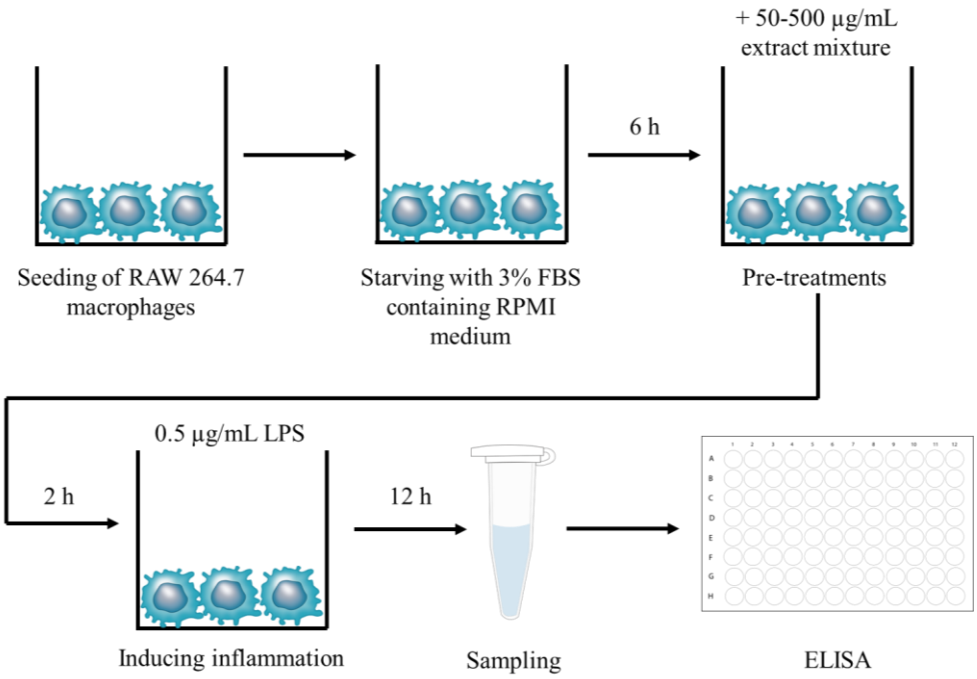

Figure 7.1. Experimental Design of *in vitro* Systemic Inflammation Model. RAW 264.7 murine macrophages were seeded in 12-well cell culture plates followed by a starving step and the pre-treatments with 50, 150, and 500 µg/mL of extract mixture. 0.5 µg/mL LPS was used as the inflammation inducer, and after 12 hours of incubation, samples were taken, and finally, the cytokine releases were determined with ELISA for mouse TNF-α and IL-6. LPS: Lipopolysaccharide, ELISA: Enzyme-linked immunosorbent assay, TNF-α: Tumor Necrosis Factor Alpha, IL: Interleukin.

7.2.5. Determination of Nitric Oxide (NO) Release

The cell density as well as the incubation period were different than that of the systemic inflammation model for the determination of NO release, therefore explained briefly in the following sentences. RAW 264.7 macrophages were seeded in $5x10^5$ cell/well density in 24-well cell culture plates and incubated for overnight attachment, as usual. Then, 50, 150, and 500 µg/mL extract mixture in FBS-free RPMI were introduced as pre-treatments

for 2 hours, followed by adding 0.5 μg/mL LPS for 24 hours. The NO release from the cells was evaluated using the Griess assay of Verdon, Burton, and Prior (1995). For that, 50 μL sample was added into 50 μL sulfanilamide prepared as 1% in 5% orto-phosphoric acid. The reaction mixture was incubated at room temperature for 10 minutes in the dark, and then 50 μL of 0.1% NED was added, followed by another 10 minutes incubation period under the same conditions. In the end, absorbances were recorded at 540 nm. Sodium nitrite was used as the standard in 0-200 μM concentration range dissolved in FBS-free RPMI.

7.2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture mediums from the treatments were utilized in the ELISA assays for each mouse or human cytokine to be detected. The only difference between the determinations was the corresponding antibodies, therefore, only one protocol was summarized in the following according to the kit instructions. Prior to assay, each sample medium was centrifuged at 300xg for 5 minutes to precipitate the cell debris. Then, 100 μL of the sample was added to coated ELISA plate for each well and covered with a sealer. The plate was incubated at 37°C for 90 minutes. After the period, the liquid was discarded, and 100 μL of diluted antibody was added to each well, followed by covering and another incubation of 60 minutes at 37°C. After the incubation, the liquid was again discarded, and the plate wells were washed with PBS three times. 100 μL of the diluted enzyme was added to each well before incubating at 37°C for 30 minutes. The last PBS washing step was applied five times before adding 90 μL of color-developing reagent and an incubation of 30 minutes at 37°C. In the last step, the reaction was terminated with 100 μL stop solution, and the absorbances were recorded at 450 nm. Standards from each corresponding kit were used to prepare standard curves, and the released cytokine concentrations were calculated using these standard graphs.

7.2.7. Colonic Inflammation Model

In the colonic inflammation model, some steps were added to the procedure of the systemic inflammation model. First, CaCo-2 cells were seeded into 12-well cell culture

plates and incubated for full confluency. Then, a post-confluency period was counted for 21 days for the differentiation of the cells. On the 18th day of differentiation, RAW 264.7 macrophages were seeded at approximately 2x10^{8} cell density on T-75 flasks for approximately 80% confluency. After the overnight attachment of the cells, the starving step was applied by changing the cell culture medium with 3% FBS containing RPMI medium. Then, LPS was added to be 0.5 µg/mL concentration in the T-75 flask and incubated for 12 h. The medium was collected from the flask and named as the inflammatory medium (IM). TNF-α was the chosen marker for the confirmation of inflammatory conditions; that is, an ELISA for mouse TNF-α was conducted to determine the difference between negative and positive controls.

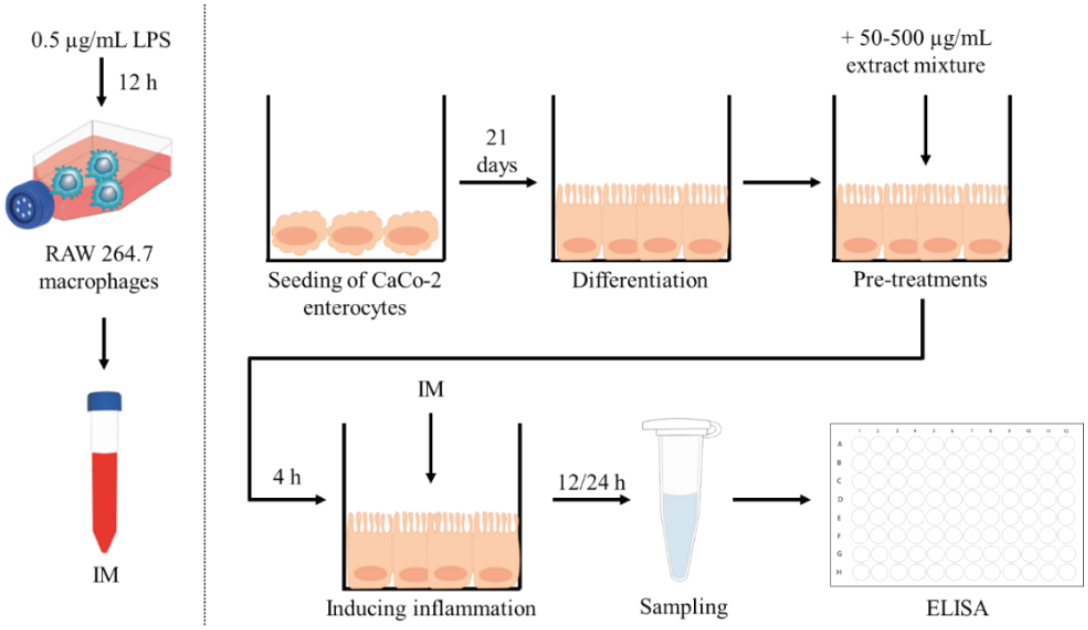

Figure 7.2. Experimental Design of *in vitro* Colonic Inflammation Model. An inflammatory medium containing inflammatory cytokines as a result of LPS treatment was used to induce the colonic inflammation in differentiated CaCo-2 enterocytes after 4 hours of 50, 150, and 500 µg/mL extract mixture pre-treatments. The released cytokines were determined with ELISA after 12 h for IL-8 and after 24 h for IL-6. LPS: Lipopolysaccharide, IM: Inflammatory Medium, ELISA: Enzyme-linked Immunosorbent Assay, IL: Interleukin

Meanwhile, the differentiated CaCo-2 cells were pre-treated with 50, 150, and 500 µg/mL extract mixture samples for 4 hours, and the mediums were discarded. Then, the IM was added as the inflammation inducer for each well and incubated for the CaCo-2 cells to release the inflammatory cytokines. Samples were taken at the end of 12 and 24 hours, and the released cytokines were determined with ELISA. Samples taken at the end of 12

hours were used for the determination of human IL-8, while the samples taken at the end of 24 hours were utilized to determine human IL-6 (Fig 7.2).

7.2.8. Statistical Analyses

All data were given as mean ± standard deviation for at least three experiments ($n \geq 3$). The samples were compared using either 2 sample t-tests or one-way analysis of variance (ANOVA), depending on the samples. Tukey's post hoc test was used for result comparisons for ANOVA ($p<0.05$).

7.3. Results and Discussion

In both inflammation models, it is essential that the applied concentration should not possess any cytotoxic activity during the experiment. Therefore, the minimum and maximum concentrations of golden thistle hyrdomethanolic extract mixture were subjected to a cytotoxicity test prior to inflammation models (Fig. 7.3). In both cell types, no cytotoxic effect was detected at 50 and 500 µg/mL golden thistle extract mixture samples ($p>0.05$). Thus, the concentration range of 50 and 500 µg/mL was determined for the systemic and colonic models of inflammation.

7.3.1. Effects on Systemic Inflammation Model *in vitro*

Figure 7.4 shows the effects of golden thistle hydromethanolic extract mixture on systemic inflammation model markers. Nitric oxide (NO) can penetrate through the cell membrane due to its lipid solubility and small size. In addition, macrophages can release significant amounts of NO during activation (Sharma, Al-Omran, and Parvathy 2007). NO is a molecule synthesized from L-arginine (Coleman 2001) and considered as an immunoregulator in low amounts, while it could be pro-inflammatory and toxic in elevated levels (Guzik, Korbut, and Adamek-Guzik 2003). Furthermore, macrophages are well-known cell types that secrete high amounts of NO in a pro-inflammatory state, making it a valuable marker for the studies (Sharma, Al-Omran, and Parvathy 2007). In

this study, the pre-treatment effect on the NO release of RAW 264.7 macrophages were evaluated in that context (Fig. 7.4a). The non-treated control sample that received neither the extract pre-treatment nor the LPS showed low amounts of NO release with 0.9 µM. On the other hand, the positive control of the experiment in which the cells were treated with 0.5 µg/mL LPS for 24 h resulted in 44.1 µM NO release. There was 49-fold difference between the negative and positive control samples of the Griess assay ($p<0.05$). And the NO releases for 50, 150, and 500 µg/mL pre-treated cells were 36.7, 30.4, and 20.7 µM, respectively. Being the lowest application, 50 µg/mL treated macrophages could not release significantly lower NO ($p>0.05$), yet a reduction was observed in a dose-dependent manner for the samples. Both 150 and 500 µg/mL treated cells showed relatively lower NO release after 2 h pre-treatments ($p<0.05$). Therefore, for the NO release assay, it can be concluded that the golden thistle hydromethanolic extracts could reduce the NO release.

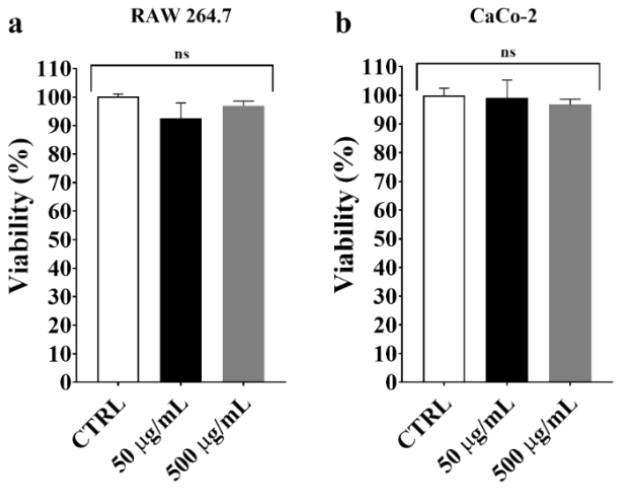

Figure 7.3. Cytotoxic Effects of the Golden Thistle Extract on the Cell Lines Used for the Inflammation Models. (a) The effect on RAW 264.7 murine macrophages used for systemic inflammation, and (b) the effect on CaCo-2 cells used for colonic inflammation models. The results are given in mean±SEM for 3 replicates (n=3). No significant cytotoxic activity was detected for 50 and 500 µg/mL extract mixture concentrations as minimum and maximum applied concentrations, as results of one-way ANOVA ($p>0.05$). CTRL: non-treated control samples, ns: not significant, ANOVA: Analysis of Variance.

Another marker of the systemic inflammation model was TNF-α. The cytokine releases were determined after 2 h pre-treatments with the extract mixture followed by 12 h of LPS application. The samples that did not get any treatments resulted in 0.85 ng/mL TNF-α release, which is relatively lower than that of the other samples as well as the positive control of the experiment. The cells that are treated with only LPS released 44 ng/mL

TNF-α, 51.8-folds higher than the non-treated control cells. For the pre-treated samples, there was a dose-dependent decrease in the cytokine release. Although 50 μg/mL concentration was not statistically significant than the LPS-treated cells, 34.47 ng/mL TNF-α release was detected ($p>0.05$). On the other hand, the samples that are treated with 150 and 500 μg/mL extract mixture resulted in 29.99 and 24.15 ng/mL TNF-α release, respectively. 150 μg/mL treatment reduced 31.8% and 500 μg/mL concentration reduced 45.1% of the cytokine release ($p<0.05$). According to these first cytokine release findings, it can be said that the golden thistle extract mixture pre-treatment was able to reduce the pro-inflammatory TNF-α release in the *in vitro* systemic inflammation model.

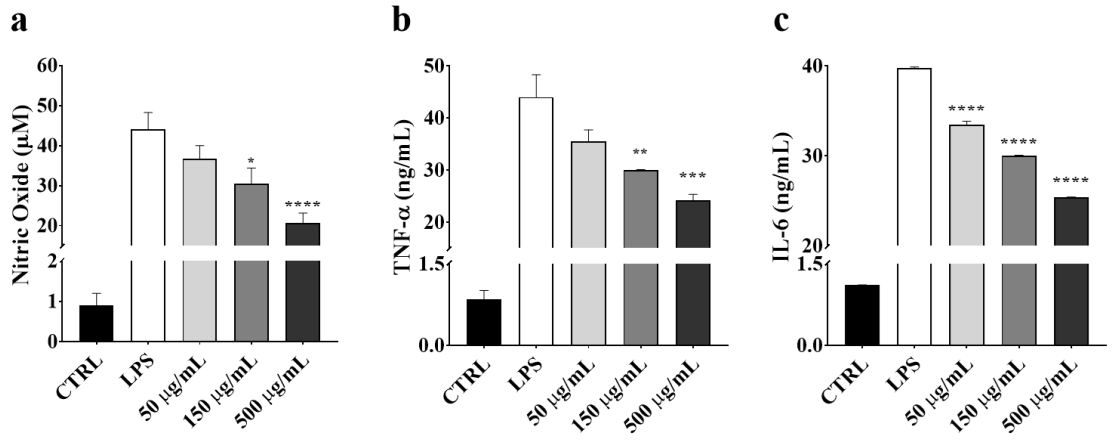

Figure 7.4. Effects of Golden Thistle Hydromethanolic Extract on Systemic Inflammation Model *in vitro*. RAW 264.7 murine macrophage cells were pre-treated with varying concentrations of the sample for 2 h and (a) nitric oxide release after LPS treatment for 24 h, (b) TNF-α release after LPS treatment for 12 h, and (c) IL-6 release after LPS treatment for 12 h were determined. The results are given in mean±SEM for 4 replicates (n=4). Statistical significances are given if the corresponding sample is different than that of LPS as the result of one-way ANOVA with Tukey's post-hoc test (*$p<0.05$; **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). CTRL: non-treated control group, TNF-α: Tumor necrosis factor alpha, IL-6: Interleukin 6, LPS: Lipopolysaccharide.

In addition to TNF-α, IL-6 is another important pro-inflammatory cytokine that is released in the early stages of inflammation. And the IL-6 releases of golden thistle hydromethanolic extract treated and non-treated samples were investigated for their IL-6 release (Fig. 7.4c). First, it is clear that the IL-6 release of non-treated control cells were very low when compared to the LPS-treated cells. The control sample resulted in 1.11 ng/mL IL-6. On the other hand, only LPS-treated cells released approximately 36 times higher IL-6 with 39.77 ng/mL. The trend of extract treated cells were similar to TNF-α as there was a dose dependent reduction in IL-6 results in the samples pre-treated with

increasing golden thistle extract mixture concentrations. 50 µg/mL concentration resulted in 33.43 ng/mL IL-6, 150 µg/mL released 29.99 ng/mL, and finally 500 µg/mL concentration was detected to have 25.36 ng/mL IL-6. The IL-6 releases of the extract treated cells were 15.9, 24.6, and 36.2% lower than the only LPS-treated cells for 50, 150, and 500 µg/mL, respectively. According to the both pro-inflammatory cytokines of TNF-α and IL-6, it can be concluded that the golden thistle hydromethanolic extract mixture might affect the inflammatory cytokine release when the RAW 264.7 murine macrophages were pre-treated with these extracts.

The production and release of pro-inflammatory cytokines are crucial during the early stages of inflammation, yet overproduction may cause serious conditions (Neurath and Finotto 2011, Strieter, Kunkel, and Bone 1993). Therefore, various plant-based molecules or their crude extracts are studied for their reducing capabilities of these two pro-inflammatory cytokines. And the plants of Asteraceae are well-known examples for their anti-inflammatory or immunosuppressive effects. Asteraceae plants such as artichoke (*Cyanara scolymus*), chicory (*Cichorium intybus*), and dandelion species (*Taraxacum* spp.) are important immunosuppressive plants (Rolnik and Olas 2021). Another example from the same family could be echinacea (*Echinacea pallida*) as it reduced the release of TNF-α significantly in human peripheral blood mononuclear cells (Senchina et al. 2011). Additionally, ethyl acetate fraction of *Ageratina picinchensis*, isolated compounds from *Artemisia halodendron* and the ethanolic extract of *Artemisia montana* reduced both TNF-α and IL-6 release in a dose dependent manner (Jeong, Kim, and Min 2018, Jin et al. 2019, Sánchez-Ramos et al. 2018). Similarly, golden thistle reduced these pro-inflammatory cytokines TNF-α and IL-6 not only in this current research, but also in the study of Berdja et al. (2021).

7.3.2. Effects on Colonic Inflammation Model *in vitro*

Colonic inflammation model was chosen as the local inflammation model as the GI tract are one of the most important cell types that meet various kinds of foreign substances, microorganisms, or toxins in the body. In this model, two cytokines were determined as the colonic inflammation markers. The first one, IL-6 is the general pro-inflammatory cytokine to be released during first stages of inflammation in macrophages, lymphocytes, and also intestinal epithelial cells (Mitsuyama et al. 1991, Mudter and Neurath 2007). IL-

6 levels elevate in the patients suffering from inflammatory bowel diseases (IBD) and cause destructive effects in epithelial layers (Nullens et al. 2016). In addition, the IL-6 release is found to be correlated with the tumor size in the patients of colorectal cancer (Becker et al. 2005). The other cytokine marker was chosen as IL-8 as being secreted as an important messenger during the colonic inflammation in the body. It has been shown that IL-8 is significantly higher in IBD patients, especially in the destructed sites (Daig et al. 1996, Mazzucchelli et al. 1994). Differently from the systemic inflammation model, the cytokine releases of CaCo-2 cell line were evaluated in the colonic inflammation. Thus, as the CaCo-2 cells are not primarily the part of the immune defense, the detected cytokine limits are relatively lower than that of the releases detected during the systemic inflammation (Fig. 7.6).

In this model, the confirmation of the inflammatory medium was done by using TNF-α as the marker. After applying 0.5 μg/mL LPS to RAW 264.7 macrophages for 12 h, TNF-α levels are checked with ELISA before inducing the colonic inflammation to the CaCo-2 cells. It was found that the LPS-treated cells released 178-folds higher TNF-α when compared to non-treated macrophages. After the confirmation, IM was given to the extract pre-treated CaCo-2 cells and the cell culture mediums were collected after 24 h.

IL-6 is the first cytokine detected with ELISA after 24 h of colonic inflammation period (Fig. 7.5a). The difference between the positive and negative control groups of the experimental conditions were 4.7-folds. IL-6 release of the control and inflammatory medium (IM) groups were 49.8 and 232.8 pg/mL, respectively ($p<0.05$). For the CaCo-2 cells pre-treated with 50, 150, and 500 μg/mL golden thistle hydromethanolic extract mixture, the IL-6 cytokine releases reduced significantly after induction of the inflammatory conditions ($p<0.05$). The IL-6 levels detected for 50 μg/mL pre-treated cells were 196.5 pg/mL, which is 15.6% lower than the IM-only treated cells. For the 150 μg/mL pre-treatment, the IL-6 release was resulted to be 196.6 pg/mL, which is a similar value for the release of 50 μg/mL pre-treatment and 15.5% lower value. And finally, the IL-6 release determined for 500 μg/mL pre-treated cells were detected as 187.4 pg/mL with a reduction of 19.5%. However, the reductions determined in IL-6 release cannot be considered as dose dependent in these experimental conditions as the cytokine values were nearly similar to one another.

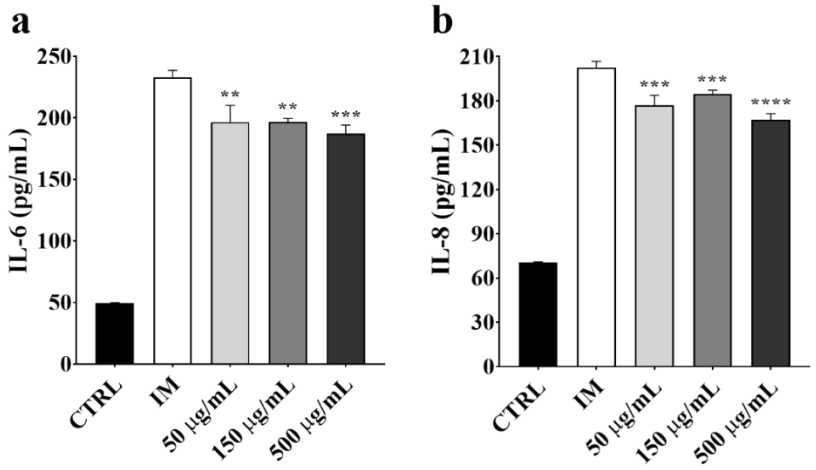

Figure 7.5. Effects of Golden Thistle Hydromethanolic Extract on Colonic Inflammation Model *in vitro*. CaCo-2 human enterocyte cells were pre-treated with varying concentrations of the sample for 4 h. Also, RAW 264.7 murine macrophages were treated with 0.5 µg/mL LPS for 12 h and the cell culture medium was collected to be the colonic inflammation inducer and named as inflammatory medium (IM). The IM were introduced to the pre-treated CaCo-2 cells as the colonic inflammation period and (a) IL-6 for 24 h and, (b) IL-8 releas after 12 h were determined. The results are given in mean±SEM for 3 replicates (n=3). Statistical significances are given if the corresponding sample is different than that of IM as the result of one-way ANOVA with Tukey's post-hoc test (**$p<0.01$, ***$p<0.001$, ****$p<0.0001$). CTRL: non-treated control group, IL: Interleukin, IM: Inflammatory Medium, LPS: Lipopolysaccharide.

As IL-8 is an important cytokine in the colonic inflammation, it was chosen as the second marker of colonic inflammation. The overall IL-8 releases of the golden thistle hydromethanolic extract mixture treated and non-treated groups are given in Figure 7.5b. However, the IL-8 cytokine releases were determined at the end of 12 h as the highest release from the positive control sample was detected in that particular timepoint. CaCo-2 cells released 70.6 pg/mL IL-8 in the non-treated control cells and this value can be considered as the basal release without any inflammatory conditions. On the other hand, the cells that are treated with IM showed 202.3 pg/mL IL-8 release. The difference between negative and positive controls was 2.9-folds ($p<0.05$). Similar to the IL-6 release, the reductions in the pre-treated enterocytes were not dose-dependent. The IL-8 releases for 50, 150, and 500 µg/mL pre-treatments were 177.0, 184.6, and 167.3 pg/mL, respectively. The reductions were 12.5, 8.7, and 17.3% in the order of increasing concentrations ($p<0.05$).

In literature, plenty of natural sources as well as isolated compounds from these sources have been shown to have preventive or healing activity in colonic inflammation. Thus,

Asteraceae plants have also been studied for their effects in colonic inflammation. For example, chamomile herbal tea reduced IL-8 release (Kogiannou et al. 2013), while dandelion (*Taraxacum* spp.), and *Rhanterium suaveolens* reduced both IL-6 and IL-8 levels *in vitro* (Ding and Wen 2018, Chelly et al. 2021). Here, the contribution of golden thistle hydromethanolic extract might contribute to the *in vitro* studies regarding both systemic and colonic inflammation models.

7.4. Conclusion: Golden Thistle Hydromethanolic Extract Reduces Inflammatory Markers in RAW 264.7 Murine Macrophages and CaCo-2 Human Epithelial Cells

According to these results and the literature data, it can be concluded that the Asteraceae member golden thistle hydromethanolic extract mixture was able to result in TNF-α and IL-6 reduction in LPS-treated RAW 264.7 murine macrophage cell line. Additionally, when CaCo-2 human enterocyte cells are pre-treated with varying concentrations of golden thistle hydromethanolic extracts, lower IL-6 and IL-8 cytokine releases were detected after induction of inflammation. Further isolation studies for the detection of biologically active molecules as well as *in vivo* studies are required to establish and anti-inflammatory effect, yet the *in vitro* cell culture models of inflammation for both systemic and colonic could be considered as the first step of the anti-inflammatory studies for golden thistle plant.

CHAPTER 8

PROLIFERATIVE AND MIGRATORY ACTIVITIES OF *Scolymus hispanicus* L. HYDROMETHANOLIC EXTRACT IN AN *in vitro* WOUND HEALING MODEL

Wounds are generally related to discomfort and reduction in the quality of life. In literature, some well-known Asteraceae members have been shown to have proliferation as well as wound healing activities both *in vitro* and *in vivo*. Thus, this section aimed to investigate whether golden thistle leads to the wound healing process, as an example of the Asteraceae family. In this part, only the root bark and root internal extracts were evaluated in terms of proliferative and wound healing activities, as the aerial part did not have any effects in the preliminary investigations. In the proliferative study, a proliferation activity test was applied to the 3T3-L1 mouse fibroblast cells. In addition, a scratch assay was conducted, calculating the migration rate and wound closures of the artificially created wounds. The results showed that the root bark extract was more effective in proliferation, migration rate, and wound closures. 80 µg/mL root bark extract showed significantly higher proliferation than the negative control group ($p<0.05$). In addition, 40 and 80 µg/mL root bark extracts showed 0.28 and 0.29 Area%/h higher migration rates after 24 h incubation. Both concentrations had nearly 39% wound closure values ($p<0.05$). A cell cycle assay was conducted using flow cytometry with the same concentrations to investigate the possible mechanism of action that the root bark extracts have. All cell cycle phases were affected by the root bark extract applications. The two higher concentrations of 40 and 80 µg/mL root bark extract resulted in 5.48 and 17.46% higher G1 phase distributions than the negative control group ($p<0.05$). In addition, these two concentrations affected the S phase and reversed the cell cycle arrest formed by reducing the S phase distributions by 16.6% and 25.3%, respectively. Lastly, there were significantly important results in the G2 phase of cell cycle distributions. All concentrations elevated the G2 phase of the fibroblasts as 5.6, 3.9, and 4.9-folds than that of the negative control groups. All in all, the root bark extract, traditionally consumed part of the golden thistle plant, showed significant proliferation and wound healing capability on 3T3-L1 cells *in vitro*.

8.1. Wound Healing Process and Plant-Based Wound Healing Aids

Skin is the largest organ in the body, mainly the combination of epithelial and connective tissue. It provides the first protection against foreign substances and other environmental effects. Therefore, any damages in the form of wounds affect the psychological status and overall quality of life. A wound is defined as "damage or disruption to the normal anatomical structure" (Velnar, Bailey, and Smrkolj 2009). Wounds can be acute or chronic. As soon as a wound is formed in a body part, the wound healing process starts with the action of various cell types. Some important cells and cell products that aid in the wound healing process can be listed as keratinocytes, fibroblasts, endothelial cells of vessels, recruited immune cells, and their extracellular matrix (Martin and Nunan 2015). Overall, wound healing comprises three main stages: inflammatory, proliferative, and remodeling (Christian et al. 2006). In the first stage of the inflammatory phase, immune cells of neutrophils accumulate in the wound area, and macrophages are activated. These two cells mark or phagocyte the microorganisms that contaminated the wound. Another critical role of the immune cells is the cytokines they release to represent the inflammatory site. During the proliferative phase, the fibroblast cells proliferate fast to eliminate the gaps occurred in the wound as soon as possible. Meanwhile, they secrete the extracellular matrix that contains glycosaminoglycan, glycoproteins, cytokines, mucins, secreted C-type lectins, galectins, semaphorins, plexins, and some modifying enzymes for crosslinking activity. The extracellular matrix is degraded and modified mainly by matrix metalloproteinases in the later stages of wound healing (Nour et al. 2019). The proliferative case starts with re-epithelization and is followed by tissue formation. There are fibroplasia and angiogenesis steps in the tissue formation step (Christian et al. 2006). The fibroblasts are responsible for wound contraction. After proliferation, they are differentiated into myofibroblasts and form wound contraction by increased collagen synthesis (Kurahashi and Fujii 2015, Velnar, Bailey, and Smrkolj 2009). Essential growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), Wnt signaling proteins, transforming growth factor beta (TGFβ), and amphiregulin function in the wound healing process (Nour et al. 2019).

As can be understood, an inflammatory phase is crucial in the wound healing process for the defense mechanism of the body (Martin and Nunan 2015). Thus, typical wounds show the five symptoms of inflammation: redness, heat, swelling, edema, and loss of function

(Velnar, Bailey, and Smrkolj 2009). However, stress-induced overproduction of inflammation markers might delay the healing significantly (Vileikyte 2007). Like in all inflammation conditions, there must be a balance between the inflammatory cytokine release.

Acute or chronic wounds can affect the overall well-being of a subject uncomfortably. Therefore, many traditional and novel applications are under investigation for this purpose. Constituting synthetic and/or natural structures is one of the important applications of bioengineering (Dickinson and Gerecht 2016). The other important application in wound healing is the usage of traditional or local medicinal plants. Plants such as *Aloe vera*, burdock (*Arctium lappa*), frankincense (*Boswellia sacra*), honeysuckle (*Lonicera japonica*), *Verbascum* species, neem (*Azardirachta indica*), ginseng (*Panax ginseng*), bay (*Wedelia trilobata*), and turmeric (*Curcuma longa*) are some of the studied plants for their wound healing potential (Budovsky, Yarmolinsky, and Ben-Shabat 2015, Sharma et al. 2021, Shedoeva et al. 2019).

As stated earlier, the wound healing mechanism is associated with inflammatory responses. Therefore, the Asteraceae plant family was not only studied but also utilized for wound healing purposes. Some of the most-known Asteraceae plants with wound healing activity are yarrow (*Achillea millefolium*), marigold (*Calendula officinalis*), chamomile (*Chamomillia recutita*), siam weed (*Chromolaena odorata*), echinacea (*Echinacea purpurea, E. angustifolia, E. palida*), sunflower (*Helianthus annuus*), and tridax daisy (*Tridax procumbens*) (Maver et al. 2015).

Asteraceae is a broad family in the plant kingdom possessing various physiological effects in extracts or other forms. Thus, this part of the thesis aimed to evaluate the possible wound healing action of the previously obtained golden thistle hydromethanolic extracts. To achieve this, an *in vitro* wound healing model was used with 3T3-L1 murine fibroblasts, and the most significant sample was evaluated for the possible mechanism of action.

8.2. Materials and Methods

In this part, 3T3-L1 mouse fibroblasts were used in the *in vitro* model of proliferation as well as wound healing models. The effects of individual root extracts on the proliferative activity were determined in the first assay. Then, a scratch assay was used to detect the

wound healing activity. Once the wound healing activity is determined, the possible mechanism of action was investigated via cell cycle assay in flow cytometry.

8.2.1. Materials and Chemicals Used

Phosphate buffered saline (PBS) tablets (P4417), Dulbecco's Modified Eagle's Medium (DMEM, D5796), penicillin-streptomycin solution (P4333), trypsin-EDTA solution (T4049), 1-(4,5-Dimetylthiazol-2-yl)-3,5-diphenylformazan (MTT, M2003), 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (propidium iodide, PI, P4170), and t-octylphenoxypolyethoxyethanol (Triton X-100, T8787) were purchased from Sigma (Mannheim, Germany). Dimethyl sulfoxide (DMSO, 102952) was from Merck (Darmstadt, Germany), and fetal bovine serum (FBS) (10270) was from Gibco (Thermo Fisher Scientific, Massachusetts, USA). Lastly, RNAse A enzyme (740505) was purchased from Macherey Nagel (Duren, Germany). Unless stated otherwise, all other chemicals used were analytical grade, and MilliQ® water was used throughout the experiments when needed.

8.2.2. Cell Maintaining

3T3-L1 mouse fibroblasts were used in all experimental models. They were maintained in 10% FBS and 1% penicillin-streptomycin containing DMEM medium at 37°C and in 5% CO_2 conditions. The passage numbers were kept between 15-25 in experiments.

8.2.3. Proliferative Activity

Cytotoxicity assay was utilized in terms of proliferative activity with an MTT assay. The detailed cytotoxicity determination was also given in Section 5.2.3. However, the seeding density, concentrations, and periods were slightly different. For the proliferative activity, the 3T3-L1 cells were seeded in 1000 cell/well density on each well in a 96-well plate. 10, 40, and 80 µg/mL of each bark extract concentrations were applied to fibroblast cells

for an incubation period of 12 h to determine proliferative activity. The rest of the experiments were the usual MTT assay as in Section 5.2.3.

8.2.4. Scratch Assay

Scratch assay can be considered as a rapid and cost-effective way of *in vitro* wound healing method (Fronza et al. 2009). In the scratch assay of the current study, 3T3-L1 cells were seeded on 12-well plates and incubated for full confluency. Then, a starving step was added to the procedure to equalize the cells. For that, 0.2% FBS containing DMEM was introduced to the cells for 16 h. At the end of the period, scratches were formed with the help of a 200 µL pipette tip. The experimental groups were as follows: the negative control cells that were treated with only 0.2% FBS containing starving medium, positive control cells that were treated with 10% complete medium, and the experimental groups of 10, 40, and 80 µg/mL of root bark and root internal sampled dissolved in 0.2% FBS containing starving medium. Once the treatments were applied, the cell culture plates were incubated in ordinary conditions until the scratch was closed in the positive control group. Microscopic photographs were taken every 3 hours for an overall experimental period of 24 h. The photographs were taken at 4x magnification from the bottom, middle, and top of each well at every timepoint.

Lastly, the microscopic photographs were evaluated on the ImageJ program with a calculation procedure from Venter and Niesler (2019). For the evaluation, the photograph was converted to grayscale, and the edges were found with the command of "find edges" in the process tool. Then, the image was blurred a few times with a "smooth" command in the process tool, and the "minimum error" function was applied by painting, adjust, auto threshold, and min error functions, respectively. In the last step, the areas were found with the command of "analyze particles" under analyze tool. The range was inserted as "size: 10,000-infinity" to achieve this. These steps result in a percentage value of the wound area. After that, two parameters, migration rate and wound closure were calculated. First, the migration rate was calculated according to Equation 8.1.

$$Migration\ Rate\ \left(\%\ Area/_{h}\right) = \frac{A_{t=\Delta t} - A_{t=0}}{\Delta t} \tag{8.1}$$

where $A_{t=0}$ is the wound area at the beginning of the experiment, $A_{t=\Delta t}$ is the wound area after 24 hours, and Δt is the experimental time of 24 h. On the other hand, the wound closure in % area was also calculated according to Equation 8.2.

$$Wound\ Closure\ (\%\ Area) = \frac{A_{t=0} - A_{t=\Delta t}}{A_{t=0}} x100 \tag{8.2}$$

The wound closure was calculated for the overall experimental period of 24 h.

8.2.5. Cell Cycle Assay

After obtaining the proliferative and wound healing activity results of the 3T3-L1 mouse fibroblasts when treated with various concentrations of root bark extract, a cell cycle assay was conducted for the determination of the possible mechanism of action. There are some similarities between the scratch and cell cycle assays. Briefly, the cells were seeded on 12-well plates and incubated for full confluency. A similar starving period for 16 h and scratching with a 200 µL pipette tip was applied for similar experimental conditions before treating the cells with golden thistle root bark extract. Then, 10, 40, and 80 µg/mL concentrations of root bark extracts were introduced to the cells. However, the experimental time was decided as 12 h, hypothesizing that the effect should have occurred earlier at the molecular level at the cell cycle steps compared to the physiologic effect under a microscope.

When the experimental period was complete, the medium in each well was taken into individual 15-mL centrifuge tubes, and the cells were washed with PBS, adding each washing PBS onto the earlier cell culture medium. The remaining cells were trypsinized and added into centrifuge tubes. When all constituents were collected, the centrifuge tubes were centrifuged at 1200 rpm for 10 minutes, and the supernatants were removed. Then, the pellets were dissolved in 1 mL of cold PBS, and 4 mL ethanol at -20°C was added into each tube while vortexing at low speed. This part was called as the *fixation*. Prior to the cell cycle assay, the fixed cells were washed with PBS 3 times, and centrifuged for 1200 rpm for 10 minutes each time. PBS was discarded after each centrifugation without touching the pellet. The washed pellet was dissolved with 200 µL of Triton X-100 (0.1% in PBS). After adding 25 µL of 200 µg/mL RNAse A to remove RNA from the reaction

medium, the tubes were incubated at 37°C for 30 minutes. Then, 25 µL of 1 µg/mL PI dye was added and incubated at room temperature for 15 minutes before analyzing the cells in a flow cytometer (Çakmak 2011).

8.2.6. Statistical Analyses

All data were given as mean ± standard error for at least three experiments ($n \geq 3$). The samples were compared by using 2 sample t-tests comparisons ($p<0.05$).

8.3. Results and Discussion

To investigate the proliferative and wound healing activity of the golden thistle hyrdomethanolic extracts, first, each extract was introduced to the 3T3-L1 mouse fibroblasts, and the microscopic evaluations were conducted. According to the screening observations, neither aerial nor extract mixture was effective on the doubling time or proliferative activity of the fibroblasts. Therefore, only root bark and root internal extracts were utilized for the experiments conducted in this section.

In the proliferative and wound healing models, first, the proliferative activity was evaluated. Then, the migration rate and wound closured were calculated with the data obtained from the scratch assay. And lastly, a cell cycle assay was conducted in a flow cytometer to investigate the effects of the golden thistle extracts on cell cycle distributions.

8.3.1. Proliferative Activity of the 3T3-L1 Mouse Fibroblasts Treated with the Golden Thistle Root Extracts *in vitro*

In the proliferation activity, 10, 40, and 80 µg/mL concentrations of root bark and root internal extracts were introduced to fibroblasts in 1000 cell/well density for 12 h. To compare the results, the absorbances obtained from the MTT assay of the positive control group in which the cells were treated with 10% FBS containing complete growth medium were considered as 99.5% viable (Figure 8.1).

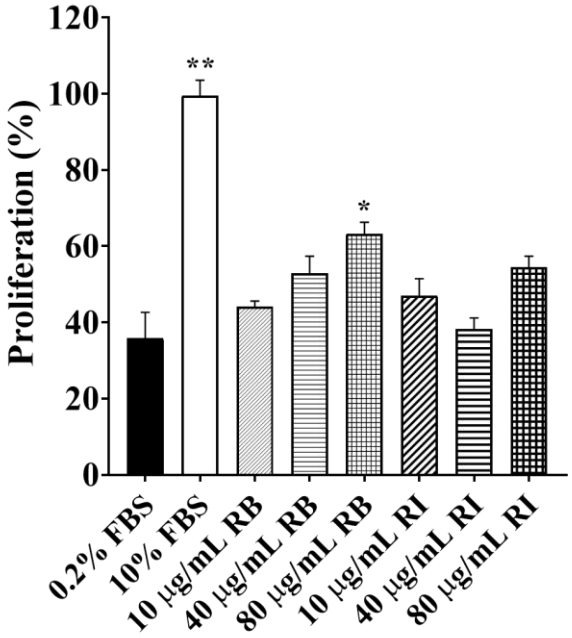

Figure 8.1. Proliferative Effects of the Golden Thistle Root Extracts on 3T3-L1 Mouse Fibroblast Cell Line. The MTT viability assay was conducted on the treated and/or non-treated cells at the end of the 12 h incubation period. The negative control group was treated with 0.2% FBS containing DMEM, while the positive control group was treated with 10% FBS containing maintaining medium of DMEM. All the golden thistle root extracts were dissolved in 0.2% FBS-containing medium before introducing to the fibroblasts. The results are given as mean±SEM in triplicate (n=3). 2 sample t-tests for each positive control and extract sample were used while comparing the results with the negative control group. FBS: Fetal Bovine Serum, RB: Root Bark, RI: Root Internal.

According to this finding, the viability of the negative control group in which the cells were treated with 0.2% FBS containing starving medium resulted in 35.8% viable at the end of 12 hours. The lyophilized extracts were dissolved in 0.2% FBS-containing medium to monitor the effects of the extracts rather than that of FBS. For the root internal samples, 10, 40, and 80 µg/mL concentrations gave 47.1%, 38.2%, and 54.4% viability values, respectively. The results for root internal samples were neither dose-dependent nor significantly different from the negative control ($p>0.05$). On the other hand, a dose-dependent increase in the viabilities of the cells treated with root bark samples was detected. 10 µg/mL root bark extract treated cells resulted in 44.1%, and 40 µg/mL root bark extract treated cells resulted in 52.9% viability. Although there was a dose-dependent increase in the fibroblast viability, the first two concentrations were insignificant ($p>0.05$). However, 80 µg/mL root bark extract treated fibroblast cells grow to 63.2% viability ($p<0.05$). Therefore, after 12 hours of the incubation period, it can be

said that the effective fraction of the root parts of the golden thistle plant was the samples from the root bark as it resulted in 1.8-folds higher proliferation than that of the negative control group.

8.3.2. Effects of Root Bark and Root Internal Extracts on Wound Healing Activity *in vitro*

Scratch assay is one of the widely used tests in the screening of wound healing activity *in vitro*. In this assay, the first 3T3-L1 fibroblast cells were seeded on 12-well plates and grown for full confluency. Then, scratches were formed manually with the help of a 200 µL pipette tip, and the cell debris was washed before introducing the varying concentrations of the golden thistle root extracts. The assay was followed by taking microscopic photographs every 3 hours. The experiment ended when a full closure of the artificial wound was detected in the positive control group. The representative photographs for each sample and time point are given in Figure 8.2.

As can be seen from the illustrations, the negative control, in which the cells were treated with 0.2% FBS containing medium, showed lower closure than that of the RB samples, and the positive control, which was the 10% FBS containing complete media given group. The proliferated cells can only be seen in the left upper part of the wound after 24 h. However, the positive control that is higher in FBS content (10%) was able to close nearly the whole wounded area during the same period. The two most effective samples of the GT plant extracts in scratch closure were detected as 40 and 80 µg/mL RB samples (Figure 8.2a). After 24 h, proliferated cells were observed at the bottom right for 40, and both right and left upper parts of the wounded area for 80 µg/mL RB samples. On contrary, root internal samples were not as effective as the root bark samples (Figure 8.2b). The wound closures were slower, and they were not fully closed at the end of the assay.

The photographs taken during the scratch assay were calculated for the wound areas by using the ImageJ program. Two findings were calculated from the results of the wound areas obtained in ImageJ as migration rate and wound closure (Fig. 8.3). The migration rates in area percentages per hour were calculated using Equation 8.2. The results showed that the negative control sample had 0.19% area/h migration while the positive control had 0.40% area/h. The 10% FBS containing medium treated positive control group had a

2.11-folds higher migration rate than that of the negative control in which the cells were treated with 0.2% FBS containing medium ($p<0.0001$). For root bark samples, the migration rates were determined as 0.25, 0.28, and 0.29% area/h for 10, 40, and 80 µg/mL concentrations, respectively. There was a dose-dependent increase in the migration rate in increasing concentrations for the root bark samples, with the two higher concentrations significantly higher than that of negative control cells ($p<0.05$). On contrary, the root internal samples resulted in 0.17, 0.22, and 0.21% area/h migration rates in increasing concentrations of 10, 40, and 80 µg/mL. None of the root internal samples gave significantly higher migration rates than the negative control group ($p>0.05$). Therefore, in terms of migration rate, it can be concluded that the root bark samples might affect the rates of fibroblast migrations in increasing concentrations *in vitro*.

Additionally, the wound closure areas in percentages were also calculated according to Equation 8.2. The results showed an approximately 2.5-folds difference between the experimental controls as the closed area was 25.31% in the 0.2% FBS treated negative control group. In comparison, the same parameter was 62.31% in the 10% FBS treated positive control group after 24 hours ($p<0.0001$). However, the overall closures were not in a similar trend to the results obtained from the calculations of migration rate. Root internal samples gave 27.76, 30.55, and 30.67% closures for 10, 40, and 80 µg/mL root internal treatments, respectively. Like in previous findings, none of the root internal treatments were significantly higher than the negative control group ($p>0.05$). 10 µg/mL root bark treated fibroblasts were also not significantly higher than the negative control with an average result of 34.16% ($p>0.05$). On the other hand, both 40 and 80 µg/mL root bark samples increased the overall closures of their corresponding wounds with 39.02% for 40 µg/mL and 38.92% for 80 µg/mL concentrations ($p>0.01$).

Regarding the calculated migration rates and wound closures, 40 and 80 µg/mL root bark treatments increased the migration rates by 32-35% and the wound closures by nearly 35%. In literature, similar plant extracts or plant-based pure molecules have been studied for their wound healing activities both *in vitro* and *in vivo*. For instance, Siriwattanasatorn et al. (2020) studied the effects of three Thai medicinal plants of *Garcinia mangostana* L., *Glycyrrhiza glabra* L., and *Nigella sativa* L. ethanolic and hydraulic extracts on 3T3-CCL92 cell line. In addition, plants like *Helianthus tuberosus* L. and *Sorocea guilleminiana* Gaudich have also been shown to have wound healing activities (Mariadoss et al. 2021, Figueiredo et al. 2020). However, the observed effects might depend on the plant material, incubation periods, and the concentrations used in the study.

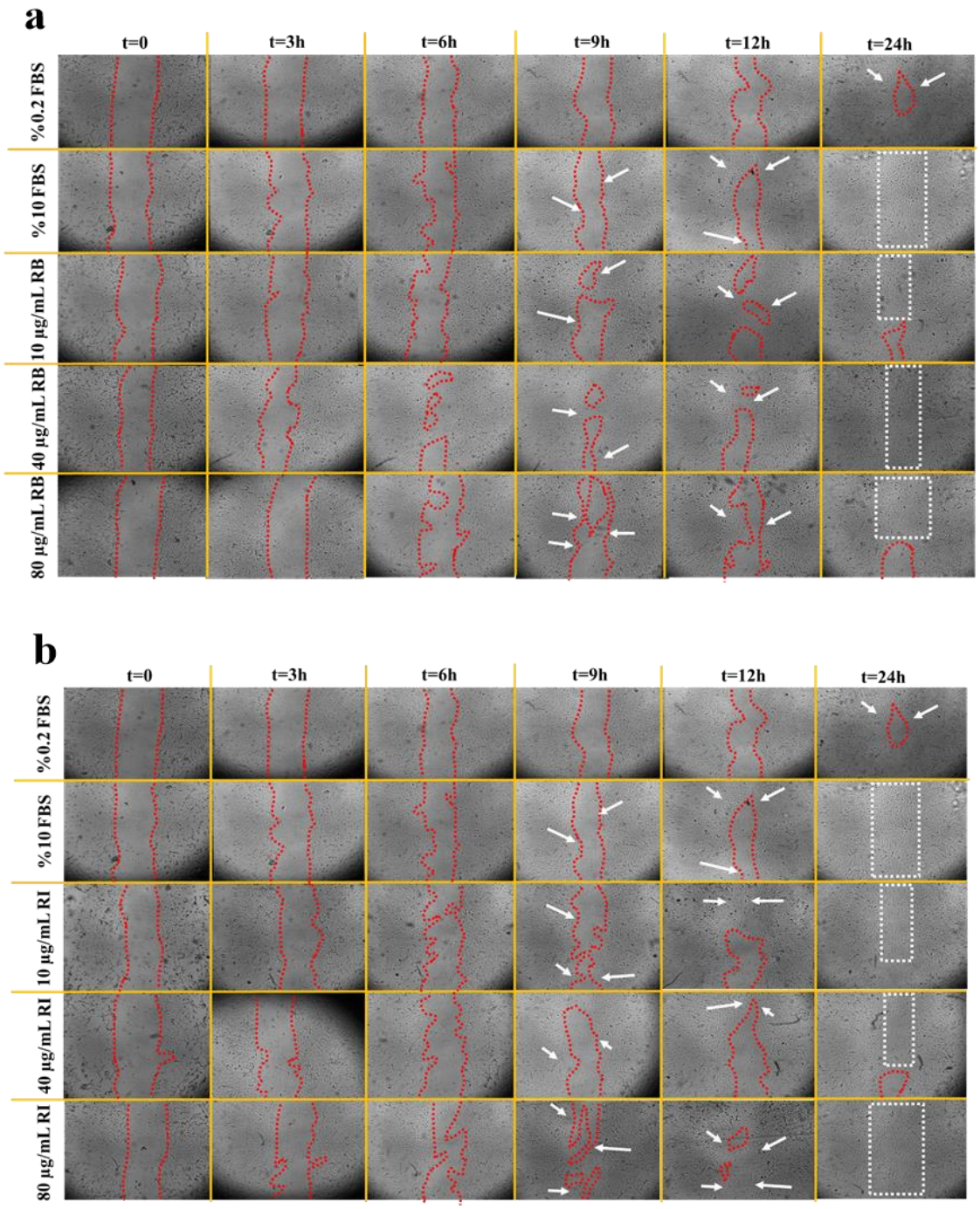

Figure 8.2. Representative Photographs of 3T3-L1 Mouse Fibroblasts of the Scratch Assay. The cells were seeded on 12-well plates, and scratches were formed artificially after full confluency. Once the cell debris was washed, a negative control medium (0.2% FBS containing DMEM), positive control medium (10% FBS containing DMEM), and golden thistle root extracts dissolved in the negative control medium were given to the corresponding wells. Photographs were taken every 3 h at 4x magnification until the wound of the positive control was fully closed after 24 h. Red dotted lines show the wound edges of the photographs, while white arrows show the closures. Additionally, white dotted rectangular representations show the fully closed areas of the wounds. FBS: Fetal Bovine Serum, DMEM: Dulbecco's Modified Eagle's Medium, RB: Root Bark, RI: Root Internal.

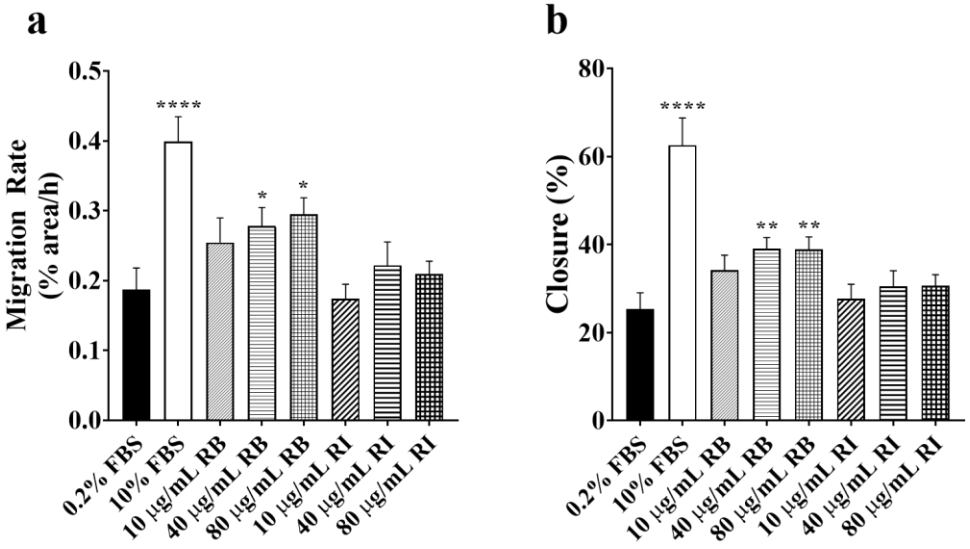

Figure 8.3. Evaluations of Wound Healing Characteristics by Using ImageJ Software. (a) Migration rate and (b) wound closure results were calculated for 3T3-L1 scratched fibroblasts after treatments of various concentrations of root bark and root internal extracts. The results are given as mean±SEM in triplicate (n=3). Statistical significance was evaluated by comparing 0.2% FBS containing DMEM treated negative control group with corresponding 10% FBS containing DMEM treated positive control group or sample extracts dissolved in 0.2% FBS containing negative control medium with 2-sample t-tests (*p<0.05, **p<0.01, ****p<0.0001). RB: Root Bark, RI: Root Internal.

The results obtained for the migration rates of the golden thistle extracts suggest that the root bark samples can be considered as effective in wound healing activity *in vitro*. Therefore, the root bark extracts of the golden thistle may be evaluated as an alternative for wound healing activity *in vitro* in terms of migration rates and wound closures.

8.3.3. Effects of Golden Thistle Root Bark Extract on Cell Cycle Distributions of 3T3-L1 Fibroblasts

In the case of wound formation, the cell cycle steps of the cells become more critical as the cells need to overcome the wound situation as soon as possible to heal (Vande Berg and Robson 2003). There are two main phases of the cell cycle: the mitotic phase, including mitosis and cytokinesis, and the interphase, which is composed of G1, S, and G2 subphases. During interphase, there are organelle and protein productions. On the other hand, there is chromosome duplication in the S phase. In summary, the cell grows in the first gap phase G1, copies the chromosomes while it continues growing in the S

phase, grows more to complete all the preparations for dividing in the second gap G2 phase, and finally divides in the M phase as shown in Figure 8.4 (Alberts 2017, Reece et al. 2011).

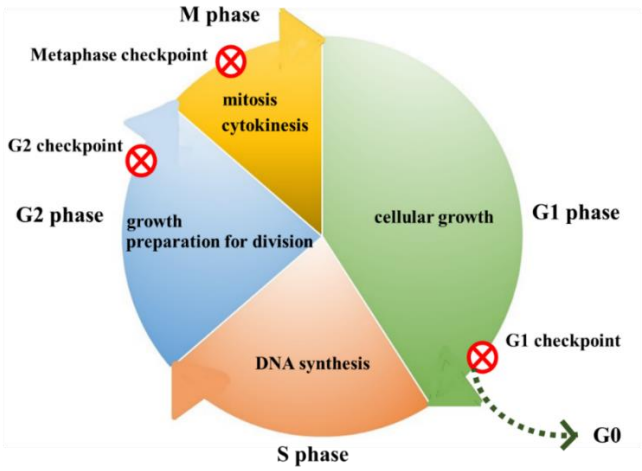

Figure 8.4. Summary Expression of the Cell Cycle. Main cell cycle phases as G1, S, G2, and M are represented with their main checkpoints and important features.

The cell cycle is controlled by the checkpoints, in other words, the cell cycle control system. There are checkpoints at the G1, G2, and M phases. Especially the checkpoint at the G1 is crucial. It is known as the restriction point in mammalian cells. At this point, if there is no signal to go further, there occurs another phase of G0, which is the nondividing phase. Cyclin-dependent kinases (Cdks) have the main role in cell cycle control. There are kinases in a growing cell, but they are generally inactive. However, if a kinase is attached to a cyclin, a maturation-promoting factor (MPF) occurs at the G2 phase, and the cell goes into mitosis. There are specific cyclins for each checkpoint as G1/S-, S-, and M-cyclins, respectively (Alberts 2017).

Then, cell cycle assay in flow cytometry was also investigated for the possible mechanism of action. For that purpose, first, the same treatment steps were applied as in the scratch assay, and then the cells were fixed, dyed, and analyzed in a flow cytometer in terms of their cell cycle distributions. The representative distribution analyses and overall results are shown in Figure 8.5.

As seen from Figures 8.5a and b, there is a clear difference between the positive and negative groups of the experimental samples. The blue-lined S phase decreased when the fibroblasts were treated with 10% FBS containing complete growth medium as the positive control. Furthermore, while there is no visible G2 phase in the negative control

group, the second and smaller red peak stands for the G2 peak of the positive control group.

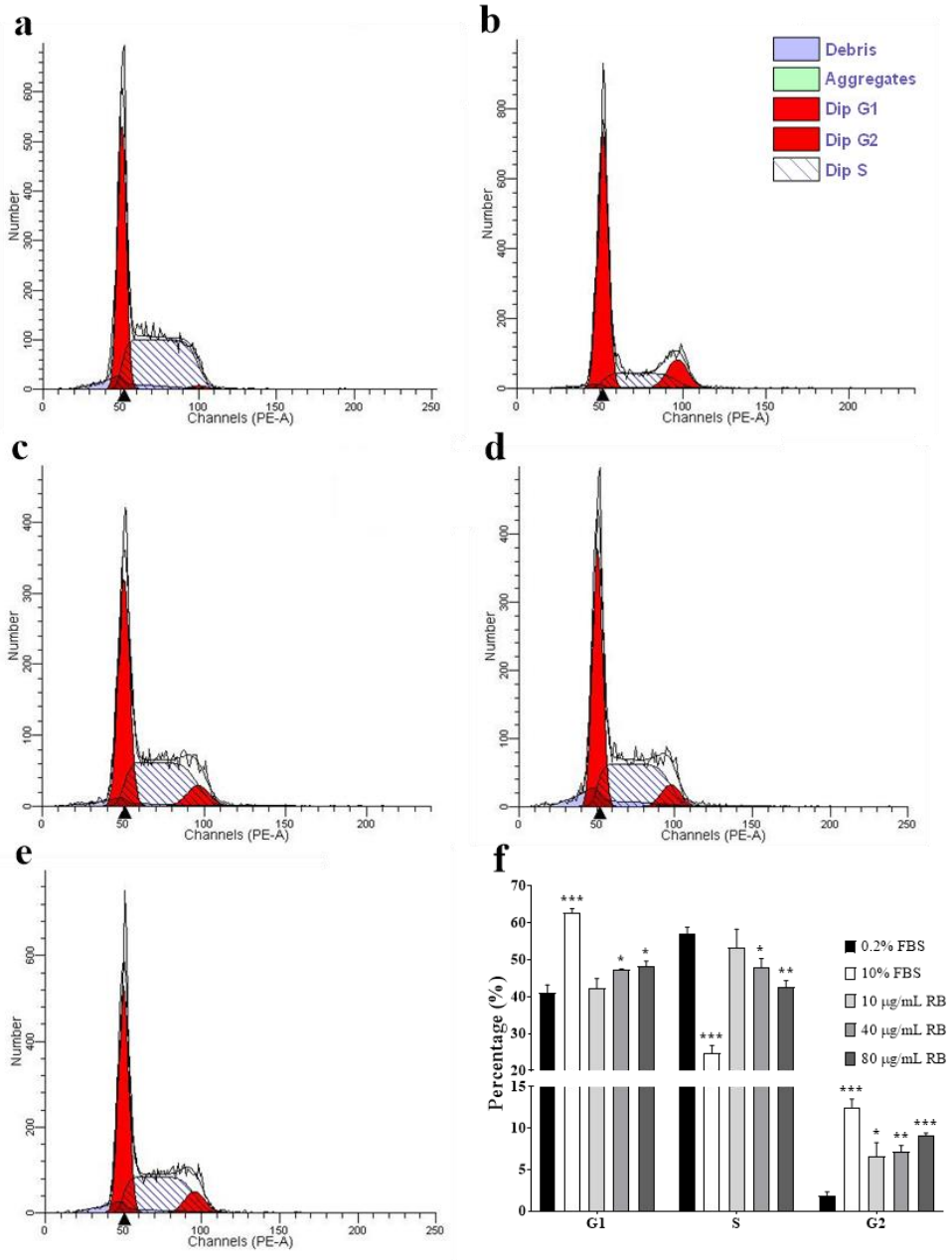

Figure 8.5. Effects of Golden Thistle Root Bark Extracts on Cell Cycle Distributions of 3T3-L1 Mouse Fibroblasts. The scratch assay procedures were repeated for 12 h of period, and the cell cycle distributions were analyzed in flow cytometry after fixation. Representative MODFIT LT™ data as the results flow cytometry of (a) 0.2% FBS containing DMEM treated cells as the negative control, (b) 10% FBS containing DMEM treated cells as the positive control, (c) 10 µg/mL, (d) 40 µg/mL, and (e) 80 µg/mL golden thistle root bark extract treatments dissolved in negative control medium. Lastly, the overall cell cycle distributions are summarized in (f). The results are given as mean±SEM for three independent replicates. The statistical analyses were conducted by 2-sample t-tests for each phase comparing the negative control and corresponding sample (*p<0.05, **p<0.01, ***p<0.001). FBS: Fetal Bovine Serum, RB: Root Bark.

Therefore, it can be concluded that treating the fibroblasts with 0.2% FBS containing DMEM caused a cell cycle arrest in the fibroblasts. For the 10, 40, and 80 µg/mL root bark treatments (Fig 8.5c, d, e), the representative data show the dose-dependent reduction in S phase and visible G2 peaks. Regarding that the root bark extracts were dissolved in the 0.2% FBS containing negative control medium, it can be commented that the phytochemicals present in the root bark extracts might cause a significant increase in the G2 phase, especially. An overall summary of the cell cycle assay revealed that the root bark extracts indeed reversed the cell cycle arrest created by the FBS shortage in the fibroblasts (Fig. 8.5f). The G1 phase distributions of the experimental groups were as follows: 41.06% for negative control, 62.67% for positive control, 42.29% for 10 µg/mL, 43.31% for 40 µg/mL, and 48.23% for 80 µg/mL root bark extracts. There was 1.5-folds difference between the negative and positive control groups ($p<0.001$). Although the 10 µg/mL root bark extract did not seem to increase the G1 distribution significantly, both 40 and 80 µg/mL concentrations were 5.48 and 17.46% higher than that of the negative control group ($p<0.05$). On the other hand, the most important differences were obtained from the S and G2 phase distributions of the cell cycle assay. The negative control cells had 57.11% S phase, which was 2.3-folds higher than the positive control value of 24.79% ($p<0.001$). This can be considered as a cell cycle arrest occurrence when the cells did not reach common growth factors present in the FBS, as the negative control group received only 0.2% FBS containing cell culture medium while the positive control cells received 10% FBS containing full growth medium. For the experimental samples of the S phase distributions, 10, 40, and 80 µg/mL concentrations resulted in 53.27%, 47.94%, and 42.69%, respectively. The 10 µg/mL concentration was again non-significant; however, higher concentrations reduced the S phase 16.6% ($p<0.05$) and 25.2% ($p<0.01$) for 40 and 80 µg/mL root bark extracts. Therefore, golden thistle root bark extract might have an important impact on the S phase distributions of the fibroblast cell cycles. The overall results revealed even more dramatic differences for the G2 phase. The negative control group had 1.84% G2 phase, while this value was 12.52%, which was 6.8-folds higher than the positive control group ($p>0.001$). The root bark extracts were all significantly higher than the negative control group with increasing distribution percentages in increasing concentrations as the G2 phase distributions of 10, 40, and 80 µg/mL root bark was 6.61%, 7.13%, and 9.08%, respectively. There were 3.5 ($p<0.05$), 3.9 ($p<0.01$), and 4.9-folds ($p<0.001$) differences than the negative control group, indicating an important feature of the effect on the G2 cell cycle phase. A similar effect on the G2 phase of the

cell cycle was observed by Madhyastha et al. (2013) using *Citrus tamurana* extract on human fibroblasts (TIG-119). Thus, it might be commented that root bark extract treated cells might pass the DNA replication of the S phase and go through the G2 phase.

The first inflammatory phase of the wound healing process includes the action of various immune cells and releases of different cytokines (Xue and Falcon, 2019). From the nutritional perspective, colonic wounds create reductions in life quality when they occur in the inflammatory bowel diseases (IBDs). For the colonic as well as various other wounds, nutritional perspective and recommendations of a high-protein diet is highly important due to the collagen release of the lamina propria fibroblasts (Reynolds, 2001; Stallmach et al., 1992). In addition, arginine is emphasized as an important requirement in tissue repair (Hadagali and Chua, 2014). Especially for colonic wounds, honey is highly recommended for its high viscosity to create a physical barrier and anti-inflammatory properties in ulcers (Hadagali and Chua, 2014). Recently, extra virgin olive oil as a source of phytochemicals was also suggested as a potential wound healing aid for colonic wounds (Melguizo-Rodríguez et al., 2021). Therefore, regarding the current results obtained from this study, it can be suggested that administration of golden thistle root bark phytochemicals might also be added to the potential materials with wound healing properties to be studied for further mechanistic and developmental approaches.

8.4. Conclusion: Golden Thistle Root Bark Extract Ameliorates Wound Healing via Reversing the Cell Cycle Arrest *in vitro*

In this section, the bark extracts of the golden thistle plant were evaluated for their wound healing activity *in vitro*. First, the root bark and root internal extracts were investigated on an MTT cell proliferation assay. The 80 µg/mL concentration resulted in higher proliferation than the negative control of the experiment. The same extracts and concentrations were also evaluated in a scratch assay model using 3T3-L1 mouse fibroblasts and resulted in similar 40 and 80 µg/mL root bark extract wound healing activity. Both concentrations resulted in higher migration rates and wound closures compared to the negative control group in the experiment. Lastly, the possible mechanism of action for this wound healing activity was investigated in flow cytometry with a cell cycle assay by using root bark extracts. All three cell cycle phases evaluated were affected by differing concentrations of root bark extracts, yet the most impactful effects were

determined for the S and G2 phases of cell cycle distributions. The results showed that both 40 and 80 μg/mL root bark extracts successfully reversed the effects of cell cycle arrest formed by reducing the ordinary amount of growth factors in the FBS. The two higher root bark concentrations resulted in higher lower S phases than the negative control group. Lastly, the elevations in the G2 phase were significant for all three concentrations in the experiment. Building on these, it can be said that the root bark portion and its phytochemicals might be considered as potent candidates for further studies in wound healing and proliferative activity studies.

CHAPTER 9

CYTOTOXIC ACTIVITIES OF *Scolymus hispanicus* L. HYDROMETHANOLIC EXTRACT ON CaCo-2 HUMAN ADENOCARCINOMA CELL LINE

Cancer is a global concern worldwide as the mortality rates increase yearly. Among these, colon cancer is the third most common cancer type. A plant-based diet is commonly recommended for the prevention and treatment of cancer cases due to the presence of plant phytochemicals. Therefore, compounds of plant origin have been investigated for inhibitory activity on cancer cells. These studies often contain various cancerous cell lines and the loss of cell viability as well as the mechanistic approaches. In this part of the thesis, the extract mixture of golden thistle was utilized to understand the effect of golden thistle hydromethanolic extracts on colon cancer cells *in vitro*. CaCo-2 human adenocarcinoma cells have been treated with 2, and 4 mg/mL of extract mixture and the cell viabilities were tested with respect to time. At the end of 72 h incubation, both concentrations considerably decreased the CaCo-2 cell viability. 4 mg/mL extract mixture treatment resulted in 78.3% lower viability compared to the non-treated control group ($p<0.05$). Then, a cell cycle assay was conducted to see if the sample phytochemicals had effects on the cell cycle. The most significant results were obtained in G1 and S phases in the 4 mg/mL concentration. There was 24.1% lower G1 phase and 38.1% higher S phase distribution, indicating a possible cell cycle arrest in the S phase ($p<0.05$). For the last parameter, an apoptosis test was conducted, and loss of cell viability was expressed at the molecular level. Again, 4 mg/mL concentration resulted in 18.5% lower alive cells ($p<0.05$). In addition, the same concentration provided 3.3-folds higher early and late apoptosis than that of the non-treated control group ($p<0.05$). Therefore, the golden thistle plant might be a candidate for the further study of possible bioactive molecules in cancer research.

9.1. Colon Cancer, Conventional Cancer Therapy, and Phytochemical Research in Cancer Prevention

Cancer is a vast epidemic problem in the modern world. The latest data about cancer statistics worldwide was available from the GLOBOCAN results (Ferlay et al. 2019). According to this data, there was an estimation of 18.1 million new cancer cases, with 9.6 million deaths from the disease in 2018. Among males, the highest cancer cases were prostate and lung, whereas the highest cases were breast, lung, and cervical cancers in females. Likewise, the latest available data in our country was from the Turkish Statistical Institute (Türkiye İstatistik Kurumu (TÜİK). In Turkey, 83,163 individuals died because of cancer, contributing to 19.7% of all deaths. Among all cancers, the group of lung, laryngeal, trachea, and bronchial cancers has the most abundancy, with 25,017 individuals as the 30.8% of the cancer cases. Colon cancer cases come next with an incidence of 6,302 deaths and 7.8% mortality (Türkyılmaz et al. 2018). Thus, cancer is becoming an even larger problem not only in our country but also in the world.

The disease starts with a single cell transformation in the body. This transformed cell proliferates and forms a tumor. If this tumor remains in its original position and does not cause significant changes in its environment, it is called a *benign tumor*, and it can be taken out easily by surgery. On the other hand, if the tumor spreads to new undamaged sites of the body and changes the tissue environment, it is called a *malign tumor*, which is considered dangerous. Cancer cells are capable of secreting signaling molecules that cause the blood vessels to grow around the tumor. If a tumor gets into the lymph vessel, it is transported into another part of the body and proliferates there, this situation is called as *metastasis* (Reece et al. 2011). Generally, it takes years for a tumor to grow before being diagnosed. Cancer disease is related to genetic changes in the code, yet a single mutation cannot be responsible for the disease (Fig. 9.1).

Cancer disease has three main classes. In the first class, the tumors arise from epithelial cells. These are called *carcinomas*, and they are the most abundant cancer type in humans. The second group, *sarcomas*, is the cancerous situation of the connective tissue or muscle cells. The third class is *leukemias and lymphomas*, in which the case is derived from white blood cells, their precursors, or from the nervous system (Alberts et al. 2008).

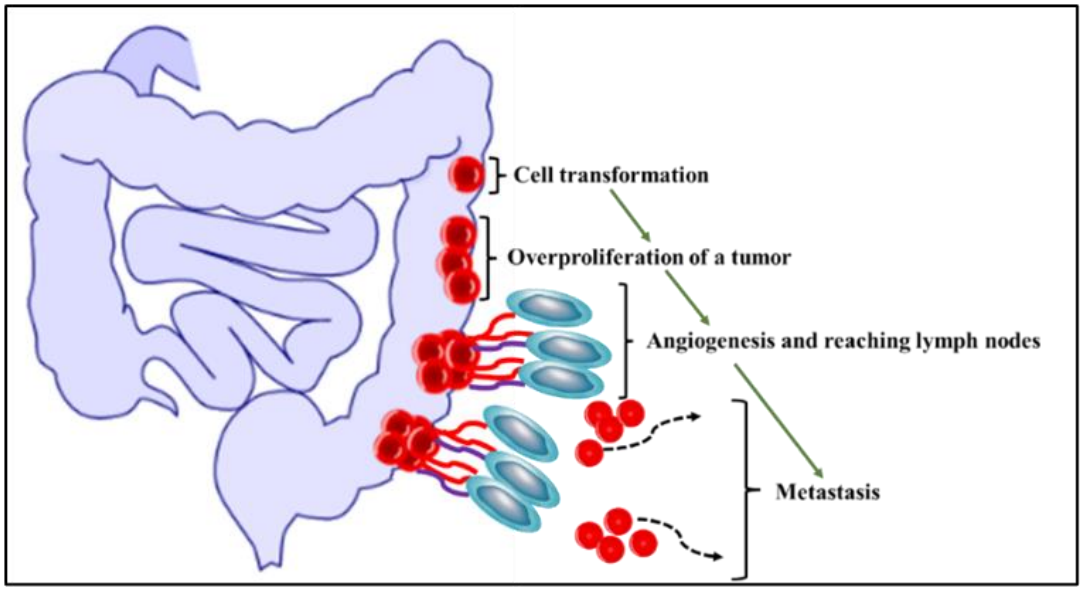

Figure 9.1. Summary of Colon Cancer Formation. The illustration of the intestinal organs was taken from ChemDraw software (Perkin Elmer, v.18.1) and combined with other demonstrations on MS Office.

In colon cancer, normal colonic epithelium turns into adenocarcinoma because of the accumulation of genetic and epigenetic alterations. Mutations in K-RAS and APC are some examples of genetic alterations. APC mutations later lead to over-activation of the Wingless/Wnt signaling pathway, which further causes chromosome instability (Grady 2004). The action in APC forms the first step named as *aberrant crypt focci* (ACF). Later, then the changes in the KRAS gene come, early adenoma occurs. In the advanced stages, mutations in TP53 and PIK3CA and loss of heterozygosity at chromosome 18q cause the final steps of late adenoma and invasive cancer (Pino and Chung 2010). Colon cancer cases show a considerable increase in the modern era due to the changes in lifestyle. Consumption of highly processed foods with less healthy ingredients, alcohol, and tobacco use are some of the factors that increase the risk of colon cancer. On the other hand, physical activity is recommended for prevention (Giovannucci 2002). A sedentary lifestyle is one of the risk factors because the colonic transit time is highly affected by the lifestyle and exercise (Gerhardsson et al. 1986). Probiotic microorganisms are also suggested for a healthy colon to reduce the risk (Wollowski, Rechkemmer, and Pool-Zobel 2001). In most cancers, the condition of inflammation takes place. For colon cancer, there are some immune cells detected near the cancerous colon cells. Cytokine release from these cells can affect the carcinogenesis in both ways by either promoting or inhibiting tumor formation. NF-κB (nuclear factor kappa b), MAPK (mitogen-activated

protein kinase), STAT3 (signal transducer and anc activator of transcription 3), and AP-1 (activator protein-1) are some of the pathways that are affected in colorectal carcinomas (Terzić et al. 2010).

Chemotherapy, radiotherapy, or the combination of these two is the treatment methods for cancer, but they have huge side effects on the status of the patient. Surgery is another cure that can only be used for non-invasive tumors. Consequently, the available cancer treatments require large patience and financial support. Thus, most of the research has been conducted on the prevention and treatment of these diseases. Rather than producing medicines for curing, the emphasis was also placed on the development of functional molecule-containing food products, beverages, and supplements to minimize the risk and expense of the treatment. The molecules of the plant origin are a large source that has been widely used in history.

Emerging interest in health and well-being of the consumers has led a vast research area of different compounds for preventing and/or enhancing the health situation. There are tremendous materials that contain various molecules that can work for this purpose. These are generally called functional molecules. The molecules that can prevent the disease and have curing effect are generally used as medicines. Lately, in literature, a huge part of the functionality studies put the account on plant-based materials that are typically considered as healthy. Among all, 25% of the commercially available medicines originated from plants (Süntar, 2020). Furthermore, 74-80% of the medications used in cancer treatment are also plant-based (Dehelean, 2021).

Phytochemicals may have the ability to affect cancer invasion and metastasis. So far, phenolic compounds, including gallic acid, chlorogenic acid, caffeic acid, carnosol, capsaicin, 6-shogaol, and 6-gingerol have been widely studied against cancer (Weng and Yen 2012). Other phytochemicals responsible for the action are carotenoids, vitamins, terpenoids, steroids, indoles, and fibers (Nishino et al. 2005). There are clinical trials with specific phytochemicals such as epigallocatechin gallate, vitamin D, genistein, resveratrol, curcumin, and retinoic acid on the cancer types of prostate, leukemia, breast, bladder, thyroid, colon, kidney, melanoma, lung, pancreatic, and multiple myeloma (Kawasaki et al. 2008). The proposed mechanisms for the anti-cancer activity are the detoxification and enhanced excretion of carcinogens, the suppression of inflammatory responses, inhibition of mitosis, and the induction of apoptosis (Johnson 2007). Furthermore, phytochemicals may aid in long-term during the prevention or reduction of

the disease and generally show fewer side effects when compared to conventional cancer therapy drugs. These properties make phytochemicals promising agents in cancer therapy. For most screening studies, the candidate compound or extract is applied to the cancerous cell lines to investigate the effects and possible mechanisms before testing *in vivo*. Thus, the CaCo-2 human adenocarcinoma cell line is widely used for evaluating a phytochemical on colon cancer. Regarding this information, this last part aimed to evaluate the effectiveness of the golden thistle hydromethanolic extract mixture on the CaCo-2 human adenocarcinoma cell line. After determining the effective concentration, the possible mechanism of action was also studied.

9.2. Materials and Methods

For the cytotoxic activity of the golden thistle plant, first, the CaCo-2 human adenocarcinoma cell line has been treated with varying concentrations of individual extracts as well as with the extract mixture. This preliminary research concluded that the extract mixture was more effective in inhibiting the CaCo-2 cell viability under a microscope. Thus, the experimental procedures proceeded by using the extract mixture. An MTT viability assay was conducted, then both cell cycle and apoptosis assays were applied to investigate the possible mechanism of action.

9.2.1. Materials and Chemicals Used

Phosphate buffered saline (PBS) tablets (P4417), Minimum Essential Medium Eagle (M4655), sodium pyruvate solution (S8636), non-essential amino acids solution (M7145), penicillin-streptomycin solution (P4333), trypsin-EDTA solution (T4049), 1-(4,5-Dimetylthiazol-2-yl)-3,5-diphenylformazan (MTT, M2003), 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (propidium iodide, PI, P4170), and t-octylphenoxypolyethoxyethanol (Triton X-100, T8787) were purchased from Sigma (Mannheim, Germany). Dimethyl sulfoxide (DMSO, 102952) was from Merck (Darmstadt, Germany), and fetal bovine serum (FBS) (10270) was from Gibco (Thermo Fisher Scientific, Massachusetts, USA). RNAse A enzyme (740505) was purchased from Macherey Nagel (Duren, Germany) and FITC Annexin V Apoptosis Kit

(640914) was from BioLegend (San Diego, CA, USA). Unless stated otherwise, all other chemicals used were analytical grade, and MilliQ® water was used throughout the experiments when needed.

9.2.2. Cell Maintaining and Treatments

CaCo-2 (HTB-37™) was purchased from American Tissue Culture Collection (ATCC®). The cells were maintained in 15% FBS containing MEM with 1% sodium pyruvate, 1% non-essential amino acid solution, and 1% penicillin-streptomycin. All maintaining and treatments were incubated at 37°C temperature with 5% CO_2 content. Passage numbers were kept between 25-35 throughout the experiments.

In all experiments, CaCo-2 cells were seeded in either 96 or 6-well plates in 4000 and $4x10^5$ cell/mL density, respectively. After waiting for an overnight attachment, a synchronization to equalize the life cycles of the cells was applied with FBS-free MEM for 16 hours. Then, 0.5, 1, 2, and 4 mg/mL of golden thistle hydromethanolic extract mixture was introduced to the cells for 72 hours in a maximum incubation time, and the corresponding experiment was conducted afterward.

9.2.3. Antiproliferative Activity

The antiproliferative activity test was applied with the help of an MTT assay in 96-well plates. Before the cytotoxicity experiment, microscopic photographs were taken as representative illustrations of the effects of different concentrations of the golden thistle extract mixture. The cells were treated with 0.5, 1, 2, and 4 mg/mL concentrations of the sample for 6, 12, 24, 48, and 72 hours. Green tea extract in 2 mg/mL concentration was utilized as a standard-like sample for the cytotoxicity assay as the effects of green tea have been well-established in literature (Esghaei et al. 2018, Ekmekcioglu et al. 1999). The continuity of the experiment was done by the previously explained MTT procedure in Section 5.2.3.

9.2.4. Cell Cycle Assay

The cell cycle assay was investigated in 6-well plates. After seeding and synchronization, 2 and 4 mg/mL concentrations of golden thistle extract mixture were applied to the CaCo-2 cells for 72 hours. Then, the assay steps of cell collection, RNAse A treatment, and dyeing with propidium iodide (PI) were applied according to the procedure as given in Section 8.2.5 using flow cytometry.

9.2.5. Apoptosis Assay

To determine apoptotic activity, CaCo-2 cells were seeded in 6-well plates, synchronized, and incubated with 2 and 4 mg/mL golden thistle extract mixtures for 72 hours. At the end of the period, an apoptosis test was executed according to the kit procedures. Briefly, the cells were washed, trypsinized, and collected in 15-mL centrifuge tubes. Then the samples were centrifuged at 800 rpm for 5 minutes, and the supernatants were discarded. Next, the pellets were dissolved in 10 mL PBS and again centrifuged under the same conditions. After removing supernatants, the remaining pellets were dissolved in 100 µL Annexin V binding buffer. 5 µL of FITC Annexin V and 10 µL of PI were added before gently vortexed. The reaction mixture was incubated for 15 minutes in the dark at room temperature, and 400 µL of an additional Annexin V binding buffer was added before the analysis in flow cytometry. The experimental controls such as experimental blank with no dyeing, only-FITC Annexin V dyeing, and only-PI dyeing were also included in addition to the experimental non-treated control cells, 2 mg/mL and 4 mg/mL extract treatments.

9.2.6. Statistical Analyses

All data were given as mean±SEM for at least three experiments ($n \geq 3$). The samples were compared by using one-way analysis of variance and Tukey's post-hoc tests ($p<0.05$).

9.3. Results and Discussion

The investigations of cytotoxic activity of the golden thistle extracts started with the applications of 0.5-4 mg/mL concentration range of individual extracts and the extract mixture by evaluating the cytotoxic phenotype under a microscope. The most significant loss of viability was obtained with the two highest concentrations of extract mixture, as shown in Figure 9.2. The non-treated control cells were nearly fully proliferated (Fig. 9.2a), while detached cells can be seen in the bottom left of the 2 mg/mL application (Fig. 9.2b). On the contrary, most of the cells were detached and showed inhibition for the 4 mg/mL golden thistle extract mixture sample at the end of 72 hours (Fig. 9.2c).

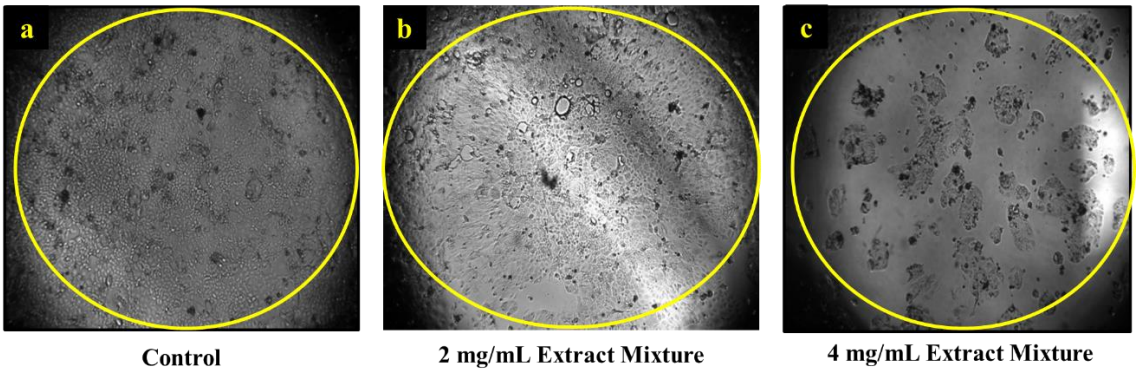

Figure 9.2. Microscopic Photographs of the CaCo-2 Human Adenocarcinoma Cells After Golden Thistle Hydromethanolic Extract Treatments. (a) non-treated control cells, (b) 2 mg/mL, and (c) 4 mg/mL treated cells under microscope. The cells were synchronized using FBS-free MEM before treatments. Then, corresponding concentrations were prepared in 15% FBS containing MEM and the incubation period was 72 hours. FBS: Fetal Bovine Serum, MEM: Minimum Essential Medium Eagle.

9.3.1. Cytotoxic Activity of the Golden Thistle Extract on CaCo-2 Human Adenocarcinoma Cell Line

The cytotoxic activity of the extract mixture was evaluated for various concentrations and periods (Fig. 9.3). When the non-treated control group was considered as 100% viable, the first 6-, 12-, and 24-hour timepoints did not result in any significant difference between the golden thistle samples (Fig. 9.3a). The viabilities calculated for 0.5 mg/mL concentration were 79.69-99.44%, for 1 mg/mL concentration were 87.86-111.83%, for 2 mg/mL concentration were 84.55-102.11%, and for 4 mg/mL concentration were 68.51-

89.13% ranges. On the other hand, a clear decline could be detected in the 2 mg/mL green tea application as the viabilities of the green tea-treated cells were 92.65, 69.66, and 74.83% for 6, 12, and 24 hours, respectively. There was also no statistical significance between the samples at the end of 24 h ($p>0.05$). In 48 hours, the viabilities started to separate from each other. 97.06, 76.72, 68.07, 35.81, and 22.00% viabilities were calculated for 0.5, 1, 2, 4 mg/mL golden thistle extracts and 2 mg/mL of green tea, respectively. An increasing trend in the viability loss was detected with increasing concentrations. 0.5 mg/mL reduced the viability by 2.94%, while 1 mg/mL reduced the value by 23.28%. 31.91% and 64.19% reductions were found for 2 and 4 mg/mL. In 24 h timepoint, the reduction in green-tea extract treated CaCo-2 cells was 78%. At the end of 48 h, only 4 mg/mL concentration was significantly lower than other concentrations ($p<0.05$).

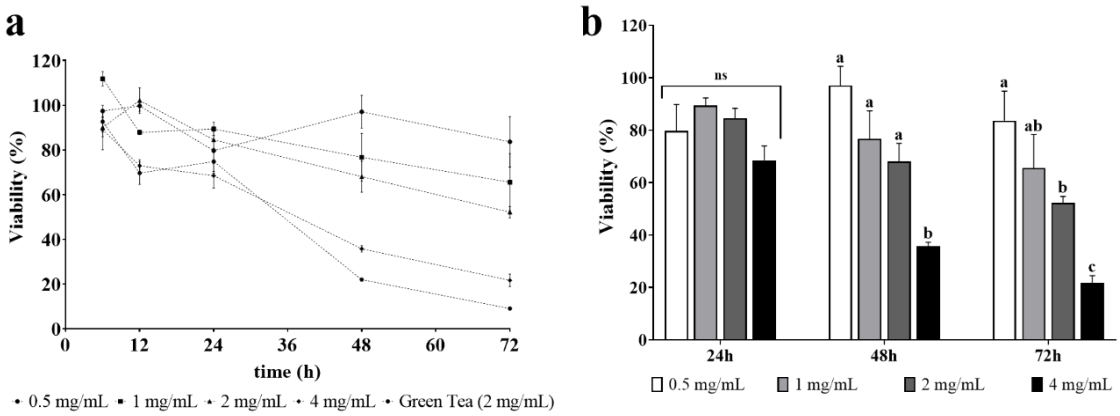

Figure 9.3. Cytotoxic Evaluations of the CaCo-2 Cells Treated with Different Concentrations of the Golden Thistle Hydromethanolic Extract for up to 72 h. (a) Decline in the cell viabilities with respect to time and (b) The histograms of the cell viabilities for 24, 48, and 72 h of incubations. The data are given as mean±SEM for at least 3 replicates ($n\geq3$). Statistical significancies were evaluated using one-way ANOVA with Tukey's post-hoc test. In section (b), different small letters on each bar represent statistical significance ($p<0.05$).

However, at the end of 72-hour incubation, the differences became clearer. 0.5 mg/mL sample was 83.65% viable with a reduced value of 16.35%. 1 mg/mL sample resulted in 65.62% viability with 34.38% reduction. 2 mg/mL sample was significantly lower than the first two concentrations with 52.21% viability and 47.79% loss of viability ($p<0.05$). Finally, the highest concentration of 4 mg/mL was calculated as 21.73% viable, causing 78.27% viability loss of CaCo-2 cells ($p<0.05$). The green tea extract group that was assumed as the positive control of the study was only 9.08% viable at the end of the experiment by reducing 90.92% of the cells.

In literature, the Asteraceae family that the golden thistle belongs to has also been studied for anticarcinogenic as well as cytotoxic activities. For instance, *Ageratum conyzoides*, *Achillea millefolium* (yarrow), *Calendula officinalis* (marigold), and *Taraxacum* spp. (dandelion) have been shown to have cytotoxic activity in cancerous cell lines (Diallo et al. 2014, García-Risco et al. 2017, Ovadje et al. 2016). Regarding other effects that the Asteraceae and golden thistle plants possess, the cytotoxic activity might be considered relatively low than the well-known examples.

9.3.2. Effects of the Golden Thistle Extract Mixture on Cell Cycle Distributions

Environmental factors such as plant phytochemicals might have impacts on the usual cell cycle distributions. In that context, plant phytochemicals might show different effects depending on the dose. For instance, the golden thistle root bark extract (RBE) showed a proliferating effect on mouse fibroblasts, as evaluated in the previous chapter (Section 8.3.2). However, in relatively higher doses, the effect on the cell cycle distribution might be reversed. The results of the cell cycle steps after treatments with the golden thistle extract mixture (GTE) are shown in Figure 9.3. When the CaCo-2 human adenocarcinoma cell line was treated with 2, and 4 mg/mL GTE, G1 and S phases of the cell cycle steps changed (Fig. 9.3a, b, c). The G1 phase in the cell cycle represents the first growing phase after division. In addition, the S phase stands for the DNA replication step. The decrease detected in both concentrations in the G1 phase might indicate that the usual growing phase was affected by the GTE phytochemicals as the non-treated control group resulted in 58.57%, while 2 and 4 mg/mL GTE treatments showed 52.75% and 44.45% G1 phase distribution, respectively (Fig. 9.3d). There were 9.9% and 24.1% reductions in the G1 phase compared to the control group for 2 and 4 mg/mL concentrations ($p<0.05$).

Among the cell cycle phases, the most significant differences were detected in the S phase. The S phase distributions for non-treated control, 2 mg/mL and 4 mg/mL GTE-treated cells were 36.36%, 36.54%, and 43.31%, respectively (Fig. 9.3d). The 2 mg/mL treatment was 16.5%, and the 4 mg/mL treatment was 38.1% higher in S phase distribution compared to the control CaCo-2 cells ($p<0.05$). Therefore, it can be commented that GTE treatments might have caused a cell cycle arrest in the S phase, indicating that the cells could not pass the checkpoint for further growth after replicating

their DNA. Finally, for the cell cycle assay, there were no significant differences between the control and experimental groups for the G2 phase ($p>0.05$). According to the cell cycle assay, it can be concluded that the GTE phytochemicals affect the cell cycle steps of CaCo-2 human adenocarcinoma cells when they are treated in 2 and 4 mg/mL concentrations for 72 hours.

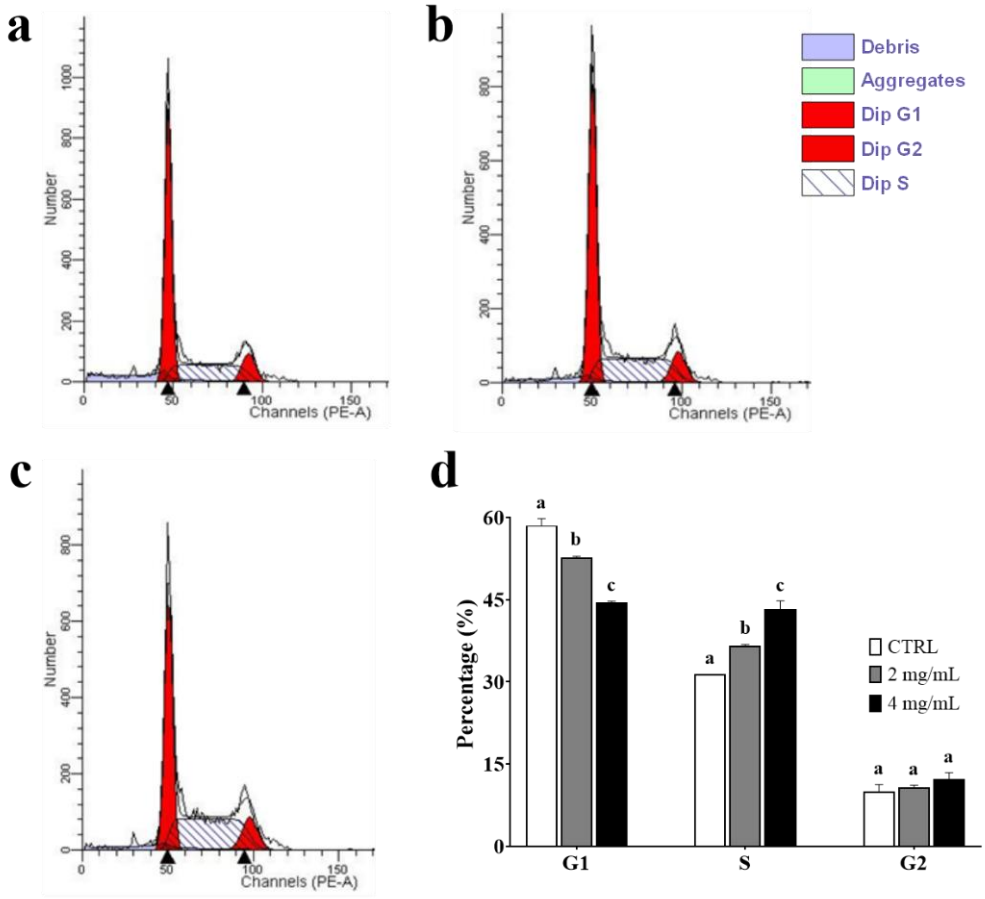

Figure 9.3. Cell Cycle Distributions of the CaCo-2 Cells After Golden Thistle Extract Treatments. The cells were treated with 2 and 4 mg/mL GTE for 72 h and then analyzed for the cell cycle distributions in flow cytometry. Representative MODFIT LT™ results for (a) non-treated control, (b) 2 mg/mL GTE, and (c) 4 mg/mL GTE treatments are shown in the first three parts. (d) represents the overall cell cycle assay results. The results are given as mean±SEM in triplicate (n=3). Different letters above each bar for each cell cycle phase indicate statistical significance of one-way ANOVA with Tukey's post-hoc test. CTRL: Control, non-treated group, GTE: Golden Thistle Extract Mixture, ANOVA: Analysis of Variance.

In literature, applications of nanoparticles targeting different cancerous cell lines have been investigated for their cell cycle distributions. For instance, glucose-capped silver nanoparticles have been reported to reduce G1 and increase S phases of cell cycle distributions of human epithelioid cervix carcinoma (HeLa) cells (Panzarini et al. 2017). Another study reported a similar effect on the human epidermal carcinoma cell line

(A431) for zinc oxide nanoparticles (Patel et al. 2016). These trends in both cell cycle steps were in accordance with the results obtained for GTE. Among the Asteraceae plants, yarrow (*A. millefolium*) hydroethanolic extract has been experimented on NCI-H460 non-small cell lung cancer as well as HCT-15 human colorectal adenocarcinoma cell lines (Pereira et al. 2018). The authors reported reductions in the G1 phase for HCT-15 cells after a treatment of 48 h. Although there was no statistical difference, the S phase was also reduced for the same cell line. However, they did not detect dramatic changes in the NCI-H460 cells after yarrow treatments. Regarding the yarrow and golden thistle extracts together, it can be said that the plant phytochemicals, including the molecular groups from the family of Asteraceae, might be evaluated as candidates for further developments in colorectal cancer treatments.

9.3.3. Effects of the Golden Thistle Extract on Apoptotic Properties

In addition to the effects on the cell cycle, plant phytochemicals might also show apoptotic properties on the CaCo-2 human adenocarcinoma cell line. Therefore, an apoptosis assay was conducted to investigate the effects of the golden thistle hydromethanolic extract mixture on flow cytometry.

As can be seen from Figure 9.4, the cells with certain properties are cluttered in one of the four regions of the dot plots. The Q1, Q2, Q3, and Q4 regions represent necrotic, late apoptotic, alive, and early apoptotic cells, respectively. The dot plots of all samples showed that most of the cells were cluttered in Q3 alive region. However, the dots cluttered in the Q4 region of early apoptotic cells were seen in high amounts with increasing concentrations (Fig. 9.4b, c). Similarly, more cells were cluttered in the late apoptotic region of Q2, and there were few necrotic cells in all experimental groups. Figure 9.4d shows the overall results of the apoptosis assay. There were 92.20, 88.78, and 75.18% alive cells for control, 2 and 4 mg/mL treatments, respectively. 4 mg/mL treated cells were 18.5% lower than the non-treated control group ($p<0.05$). Furthermore, the non-treated control group, 2 and 4 mg/mL golden thistle treatments, resulted in 1.60, 2.76, and 5.28% early apoptotic cells, respectively. Similarly, late apoptotic cells for the treatments were 5.60, 7.92, and 18.40%, respectively. Both early and late apoptotic distributions for 4 mg/mL concentrations were 3.3-folds higher than that of the non-

treated control group ($p<0.05$). Necrotic cells were the lowest of all experimental groups, yet 4 mg/mL treatment was significantly higher. Non-treated control cells had 0.56%, 2 mg/mL treated cells had 0.52%, and 4 mg/mL treated cells had 1.12% necrotic cells. 4 mg/mL concentration was 2-folds higher than the control ($p<0.05$).

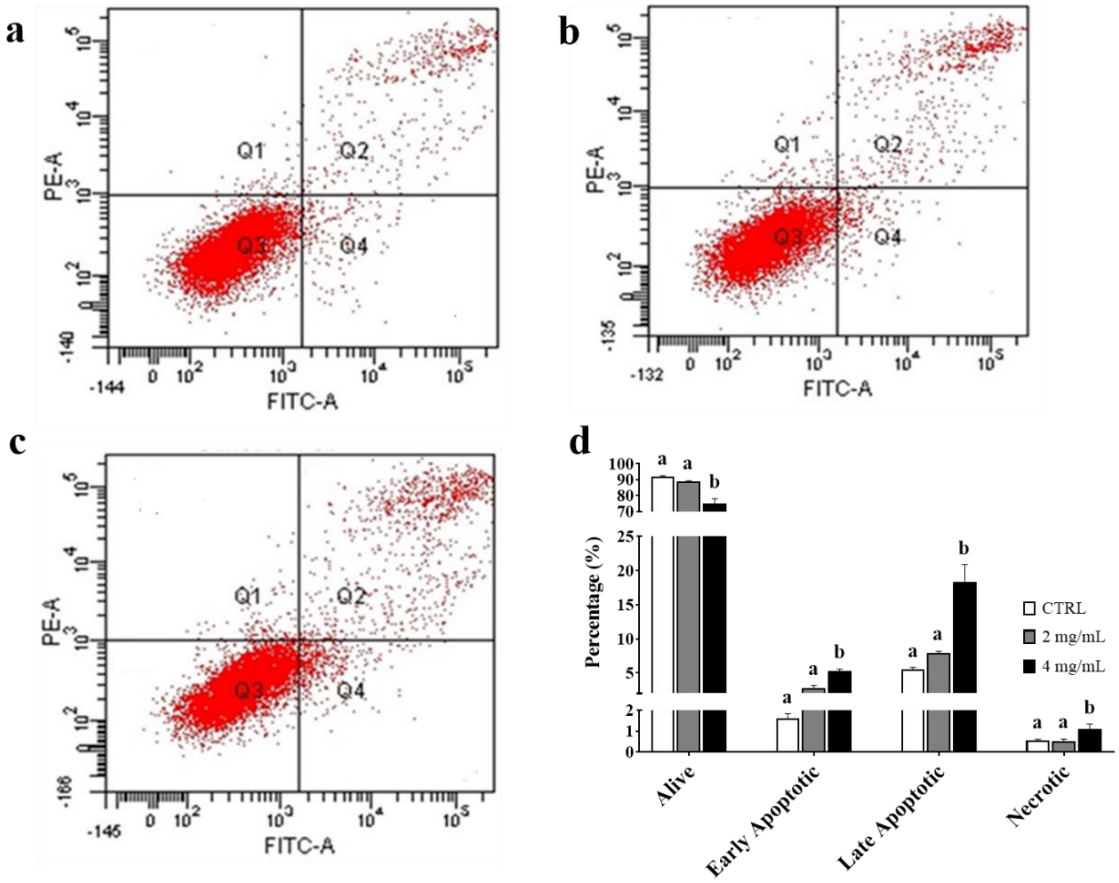

Figure 9.4. Apoptotic Activity of CaCo-2 Cells After Applying Golden Thistle Hydromethanolic Extract. The first 3 sections represents dot plots of apoptosis assay in flow cytometry for (a) non-treated control cells, (b) 2 mg/mL, and (c) 4 mg/mL extract applications. Section (d) is the summary figure of the overall apoptotic activity of CaCo-2 cells after 72 hours of extract incubation. The results are given as mean±SEM for 3 replicates (n=3). Different letters above each bar represent statistical significance of the corresponding histogram as the results of one-way ANOVA with Tukey's post-hoc test. CTRL: non-treated control group.

Various plants from the Asteraceae family have been investigated on cancerous cell lines. For example, *Vernonia cinerea* L. dichloromethane fraction has been reported to be effective on human cervix adenocarcinoma (HeLa), lung carcinoma (A549), mammary gland adenocarcinoma (MCF-7), and CaCo-2 cells (Appadath Beeran et al. 2014). In addition, extracts or isolates from *Inula helenium*, *Eucanna biolide*, *Achillea millefolium* L, and *Taraxacum* spp. induced apoptosis on acute myelogenous leukemia (KG1a), MCF-7, and colorectal adenocarcinomas cells of HCT-15, HT-29, and HCT16 cell lines,

respectively (Appadath Beeran et al. 2014, Pereira et al. 2018, Abuali et al. 2021, Ding et al. 2019, Ovadje et al. 2016). Golden thistle hydromethanolic extract might be an addition to showing apoptotic activity on the CaCo-2 human adenocarcinoma cell line as apoptotic activity and cell cycle arrest were detected after treatment (Fig. 9.5).

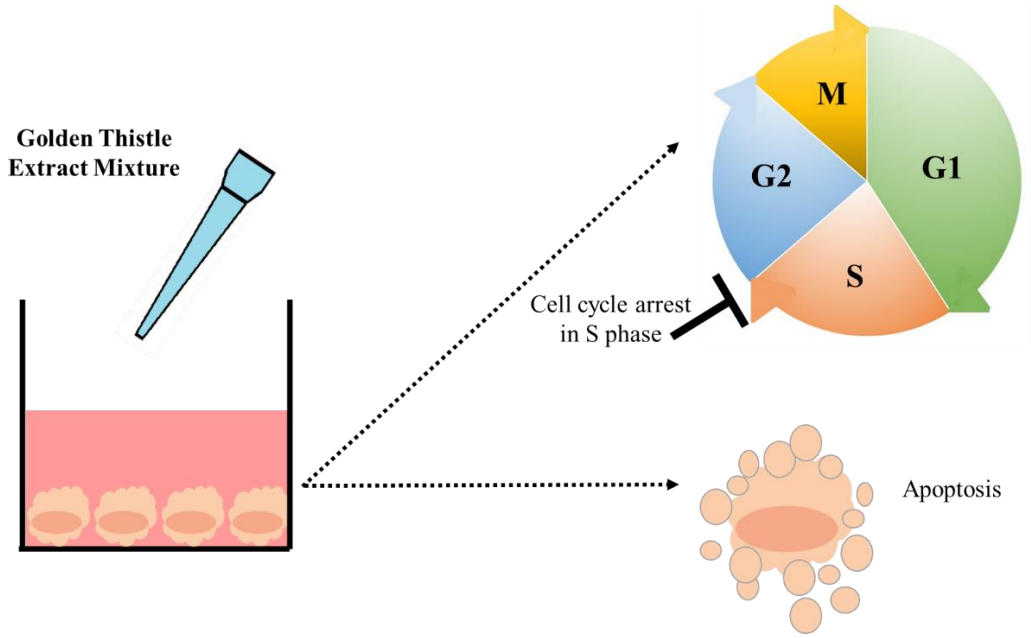

Figure 9.5. Summary Representation of the Cytotoxic Activity of the Golden Thistle Extract Treatments on CaCo-2 Human Adenocarcinoma Cell Line.

9.4. Conclusion: Golden Thistle Extract Treatment Promotes Early and Late Apoptosis in Human Adenocarcinoma Cells

Altogether, the golden thistle hydromethanolic extract mixture showed cytotoxic activity in human adenocarcinoma cell line (CaCo-2) in relatively higher concentrations. The cells were treated with 2 and 4 mg/mL concentrations. There was a time-dependent reduction in the cell viabilities for both concentrations used. After 72 h of treatments, viability loss of the CaCo-2 cells was detected. Moreover, the higher concentration in this part resulted in S phase cell cycle arrest and early and late apoptotic properties for CaCo-2 cells. Thus, the golden thistle plant might be another Asteraceae alternative in the prevention of colon carcinoma after the bioactive compounds are isolated and tested *in vivo*.

CHAPTER 10

CONDLUDING REMARKS

In this dissertation, a scanning study was applied to the golden thistle (*Scolymus hispanicus* L.) plant, which is native to the Mediterranean and Macaronesian regions. And in this last chapter, a summary of overall applications and conclusions are presented.

The golden thistle plant has three main parts. However, only the root bark part with some of the fresh raw aerial leaves are considered edible for consumers. It was used in local medicine for decades, and more importantly, the absolute ethanolic extract of the fresh plant parts was once utilized as a licensed medicine. In 2015, the Turkish General Directorate of Agricultural Research and Policies executed the cultivation of this wild edible plant in Menemen, İzmir, and provided the three parts of aerial, edible root bark, and lastly, the rigid root internal parts of the golden thistle individually. These samples were transported in dry powdered forms to Izmir Institute of Technology, Department of Food Engineering, to be utilized as the primary material in this current research.

The first step was to apply proximate analyses to the plant powders. For that purpose, moisture, protein, soluble carbohydrate, total lipids, crude fiber, and ash content analyses were performed. Among the most important outcomes of this first application, it can be said that the edible root bark portion has the lowest protein content. On the other hand, the same root bark powder was relatively higher in soluble carbohydrates. Regarding the crude fibers, the dry powdered sample of the thorny aerial part of the plant had significantly high crude fiber content. This property might be attributed to the presence of lignin in the mature harvested plant. No important lipid or ash contents were detected in the samples; thus, they are not included in the summary section. For this step, it can be hypothesized that the golden thistle plant was moderately beneficial in macronutrient contents, yet the possible micronutrients in terms of phytochemicals in the plant matrix might be considerable.

Building onto this, a hydromethanolic extract was obtained from the golden thistle powders. Each extract of aerial, root bark, and root internal were prepared individually and sometimes used in their individual forms or as a mixture sample of equal amounts depending on the preliminary studies of the corresponding experimental approach.

After obtaining the extracts, they were subjected to a thin layer chromatography (TLC) in apolar, moderately polar, and polar mobile phases. As methanol and water were the two solvents to extract the phytochemicals in the golden thistle powders, the results of the TLC showed polar retention factors, as expected. Then, total phenolic, total flavonoid, and tannin contents in the extracts were determined. Excluding the extract mixture sample, the aerial sample had the highest total phenolic and flavonoid content, followed by root internal and root bark samples. Contrarily, there were no detectable condensed tannins in the lyophilized extracts.

The following approach was to determine the antiradical capacities of the extracts. Similarly, when the extract mixture sample is excluded, the phenolic-rich aerial sample resulted in the highest Trolox Equivalent Antioxidant Capacity (TEAC). For this assay, both root bark and root internal extracts were lower than the aerial sample, with no difference. On the other hand, for the DPPH radical scavenging and Ferric Reducing Antioxidant Power Assays (FRAP), the aerial sample had the highest antiradical activity, followed by root internal and root bark.

Although the edible root bark sample did not show high macro- or micronutrient contents in advance, all extracts were combined as a mixture sample in equal volumes, hypothesizing that the different plant parts might have activities in the experimental model of the study. For that reason, the phenolic compound profile was aimed to be enlightened with High-Pressure Liquid Chromatography (HPLC). 5-*o*-caffeoylquinic acid (chlorogenic acid, CGA) was the most abundant phenolic compound in the extract mixture. This data was in accordance with the literature as CGA is one of the main phenolics in the family of Asteraceae, to which the golden thistle plant belongs to. Using a bicameral insert membrane system, CaCo-2 human enterocyte cells were grown and differentiated to mimic the human absorption in the first cell culture model. The extract sample in 1 mg/mL concentration was introduced to the apical side, which represents the gastrointestinal tract (GI) where the food matrixes are digested, and the CGA content in the basolateral part, which stands for the bloodstream where the compounds are transferred after absorption was detected with HPLC. The *in vitro* CGA bioavailability was not found to be significantly higher as expected, however, formulations and further developments might be applied to the extracts to enhance the bioavailability of CGA in the golden thistle.

As the CGA is well known for its antidiabetic and antilipidemic activities, an *in vitro* antidiabetic approach was developed to be tested for the effects of the golden thistle

extract mixture. In the first part of this study, the golden thistle extract mixture was combined with starch solution and subjected to a test tube digestion procedure mimicking mammalian digestion. However, no difference was observed between the control starch sample and the extract containing digested sample at the end of the experiment. Therefore, although the enzymatic inhibition studies are widely applied to various extracts or pure compounds in literature, the results might not be exactly the same as in mimicked digestion conditions when different enzymes and other aspects of the digestion are included. After concluding the non-remarkable effects in starch digestion, the effects on pure glucose absorption were aimed to be detected. In this second part of the antidiabetic activity study, a similar bicameral insert membrane system was used as the growing base of the CaCo-2 human enterocytes. After differentiating the cells in these membranes, the cells were pre-treated with an extract mixture, and the application medium was discarded before the glucose application in the first sample. However, the extract was co-treated with glucose in the second sample simultaneously. In both pre- and co-treatments, the same 1 mg/mL extract mixture was used. The overall results of the glucose efflux study revealed that the pre-treated sample resulted in significantly lower glucose efflux after 2 h of the experimental period. The same significant difference was also obtained as the results of the area under curve values of the glucose efflux data with respect to time. In addition, the possible mechanism at the molecular level was evaluated by subjecting the cells of the glucose efflux study to mRNA expressions. In that assay, significantly less GLUT2 mRNA expressions were observed for the GLUT2 mRNA levels for both pre- and co-treated CaCo-2 cells.

As most diseases are strongly linked to inflammation and inflammation-related complications, two models of inflammation were also included in this scanning study. In the first one, the effects of the extract mixture were tested in a systemic inflammation model using RAW 264.7 murine macrophages with lipopolysaccharide (LPS) as the inflammation inducer. For the systemic inflammation model, 50, 150, and 500 μg/mL concentrations were pre-treated with macrophages for 2 h before the LPS induction of 12 or 24 h. 150 and 500 μg/mL samples could reduce the nitric oxide (NO) levels of Griess assay and another critical inflammatory cytokine, tumor necrosis alpha (TNF-α) by Enzyme-Linked Immunosorbent Assay (ELISA). In addition, all concentrations could reduce the levels of the second pro-inflammatory cytokine, interleukin 6 (IL-6).

The GI tract is one of the most vulnerable body parts to toxins, other foreign compounds, and microorganisms. Although there are some types of defense mechanisms against these

threats in the GI tract, it can still be inflamed with a massive increase in the pro-inflammatory cytokine release, causing inflammatory bowel diseases (IBDs). Therefore, the second inflammation model was chosen as the colonic inflammation as a model for the local inflammations utilizing CaCo-2 cell line. The same concentrations of 50-500 μg/mL pre-treatments were applied for 4 h. On the other hand, RAW 264.7 macrophages were induced with LPS to obtain a cytokine mixture to be applied as the inflammation inducer to the CaCo-2 cells. In the colonic inflammation model, all pre-treatments significantly reduced pro-inflammatory cytokine releases of IL-6 and IL-8 by ELISA for 24 and 12 h of inflammatory periods, respectively.

Phytochemicals might alter the cell proliferations depending on the dose. In high concentrations, they may inhibit normal cell growth while ameliorating the proliferation activity in low concentrations. Originating from this view, the first individual extracts were applied to 3T3-L1 mouse fibroblasts in a proliferative activity model at low concentrations. Although all extracts have been included in the preliminary studies, the aerial extract did not show any proliferative activity and was therefore excluded from this model. 10, 40, and 80 μg/mL root bark and root internal extracts were applied first to the fibroblasts, followed by a viability test aiming to detect the proliferative activity. 80 μg/mL root bark extract resulted in significant proliferative activity among the samples compared to the non-treated negative control cells. The proliferative activity proceeded with a wound healing assay in which the fully confluent fibroblasts are artificially wounded. The results of the wound healing model showed that both 40 and 80 μg/mL root bark samples resulted in increased wound healing and closure values. However, the root internal extracts were ineffective in neither proliferation nor wound healing models. Therefore, only root bark extract samples were included to investigate the possible mechanism of action. For that purpose, 10-80 μg/mL root bark samples were introduced to the same wound healing model, followed by a cell cycle assay with flow cytometry. Cutting the growth factors, a cell cycle arrest was caused in the fibroblasts, and 40 and 80 μg/mL concentrations reversed this arrest by showing higher G1 phases with reductions in S phases in flow cytometry. In addition, all concentrations resulted in higher G2 phases, indicating an effect on the proliferative activity in the cell cycle.

Lastly, relatively higher doses were utilized in the cytotoxic activity study. For that model, CaCo-2 cells were used as human adenocarcinoma cells for 0.5-4 mg/mL concentrations of extract mixture samples. Similar viability tests were applied to the 72-hour-treated CaCo-2 cells. The two highest concentrations of 2 and 4 mg/mL reduced the

CaCo-2 viabilities significantly. Therefore, these two concentrations were used again to investigate the possible mechanism of action with flow cytometry. In the first assay, a cell cycle test was conducted. The results showed the opposite trend in the proliferative activity; that is, both concentrations reduced G1 proliferative phases and increased the S phases significantly. However, there were no changes in the G2 phases. These cell cycle assay results might be commented that higher doses of golden thistle hydromethanolic extract mixture applications might cause a cell cycle arrest by preventing the cell from passing into the G2 proliferative phase after replicating its DNA in the S replication phase. Furthermore, an apoptosis test was also applied to the same treatments. According to the results obtained from the apoptosis assay of flow cytometry, only 4 mg/mL sample showed apoptotic properties. There were significantly fewer alive cells in the 4 mg/mL treatments. In addition, 4 mg/mL concentration showed higher percentages of early and late apoptotic cells. Although there were significantly higher necrotic cells after 4 mg/mL treatments, the overall necrotic cells were not high compared to the alive and early and late apoptotic cells. This result indicates that the 4 mg/mL caused programmed cell death with less cell lysis, which is seen in necrosis.

All in all, the golden thistle hydromethanolic extract has been subjected to various disease and cell culture models in this study, and promising results have been obtained. Among the *in vitro* models that have been investigated, the antidiabetic activity and the effects detected in both systemic and colonic models of inflammation and proliferative activity that the root bark extract possessed were considerable. Therefore, by completing a scanning approach on this crude extract, it can be commented that identifying and isolating the possible bioactive molecules other than chlorogenic acid might be considered for further studies. The effects of the crude extract on these models also make the material a promising raw material for designing targeted formulations that include novel techniques and then investigating these preliminary results *in vivo*. Eventually, the golden thistle and the phytochemicals of this plant may have become additional bioactive materials of the treasured Mediterranean.

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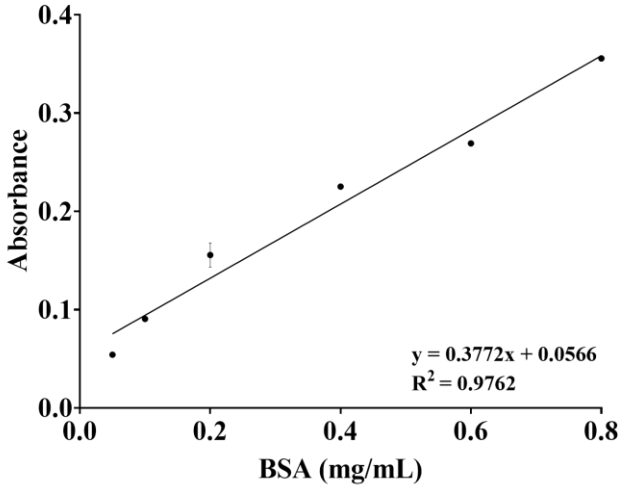
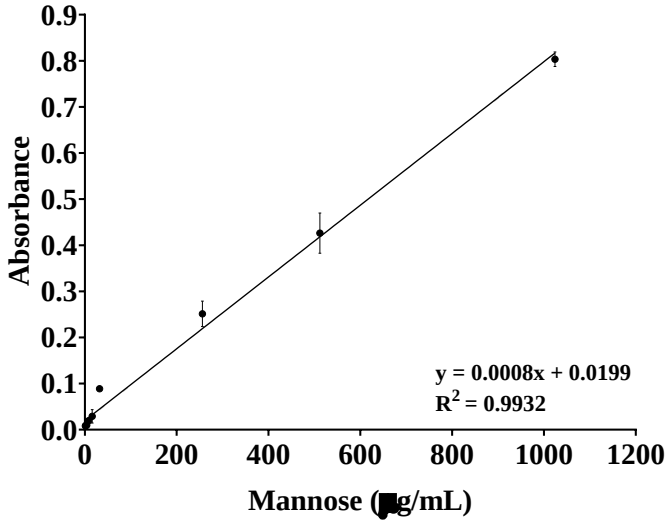
APPENDICES

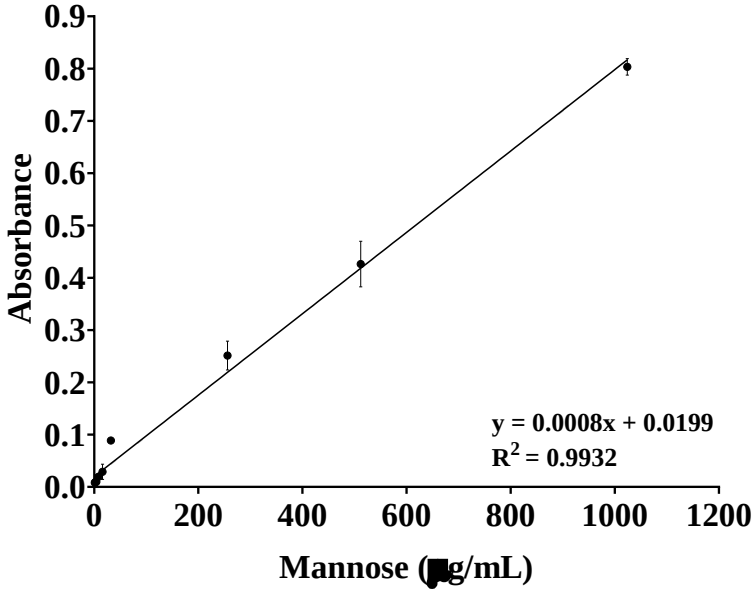
APPENDIX A.1. Bovine Serum Albumin (BSA) Standard for Bradford Assay



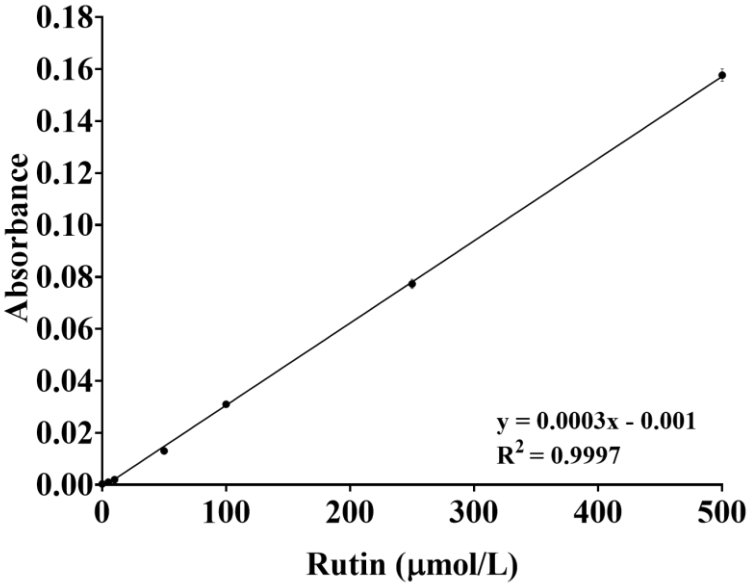
APPENDIX A.2. Mannose Standard for Soluble Carbohydrates Assay



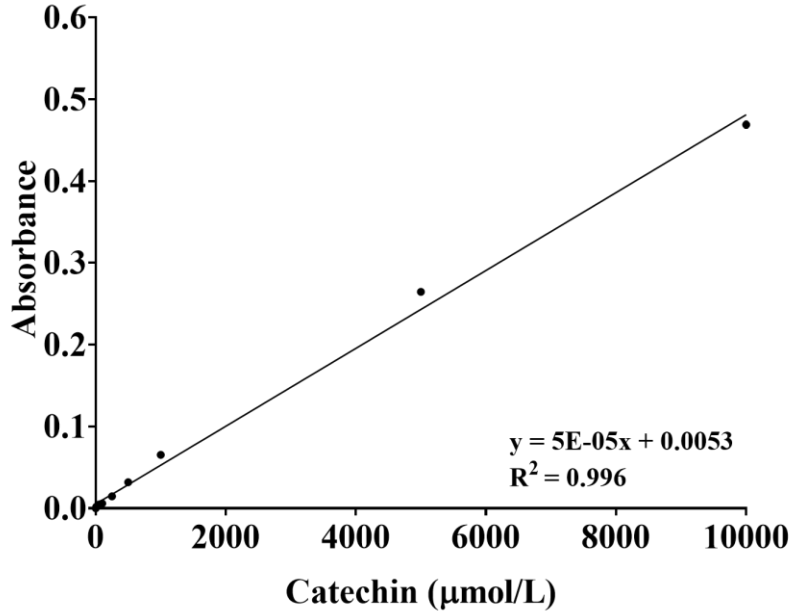
APPENDIX A.3. Gallic Acid Standard for Total Phenolic Content Assay



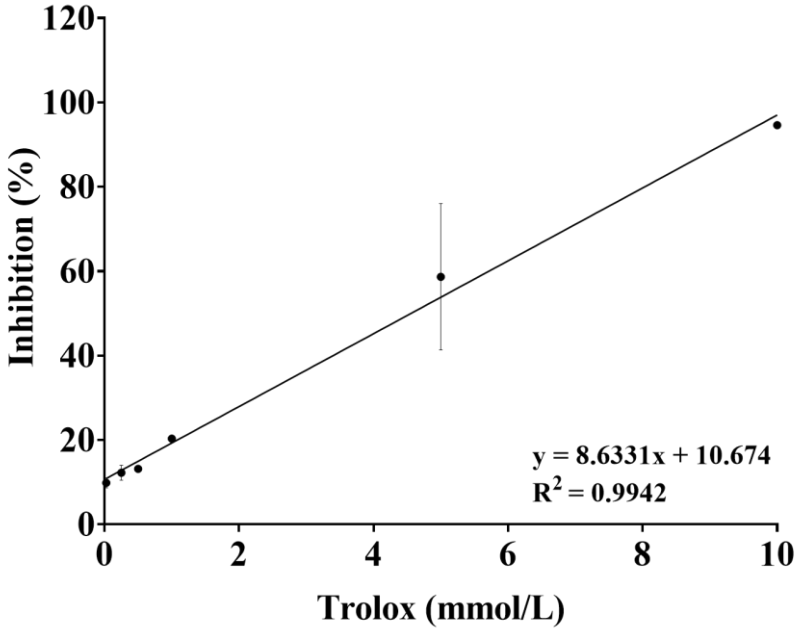
APPENDIX A.4. Rutin Standard for Flavonoid Content Assay



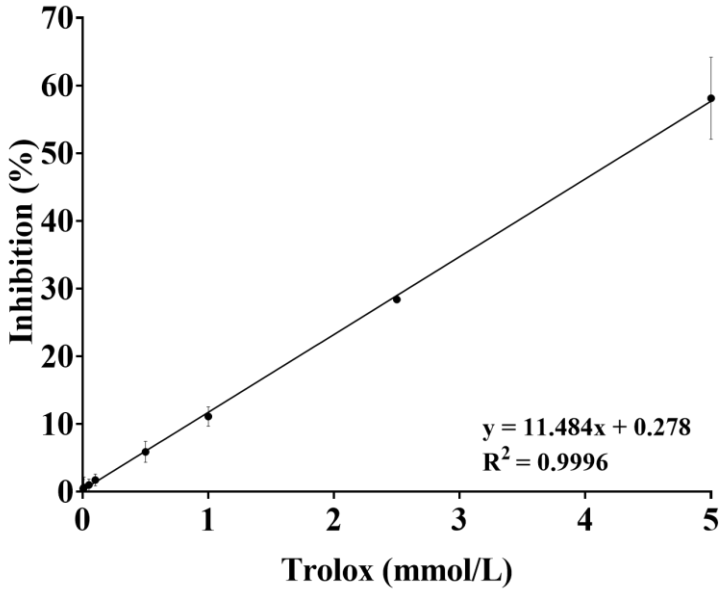
APPENDIX A.5. Catechin Standard for Flavonoid Content Assay



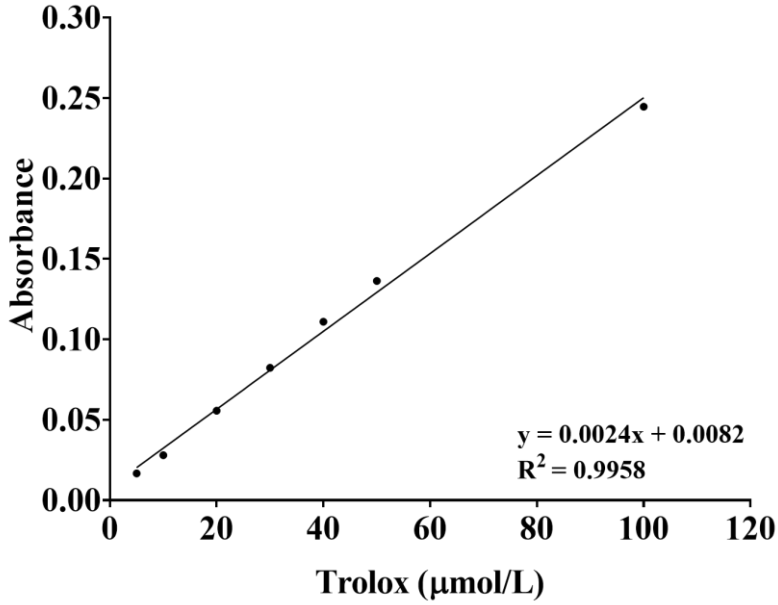
APPENDIX A.6. Trolox Standard for Trolox Equivalent Antioxidant Capacity (TEAC) Assay



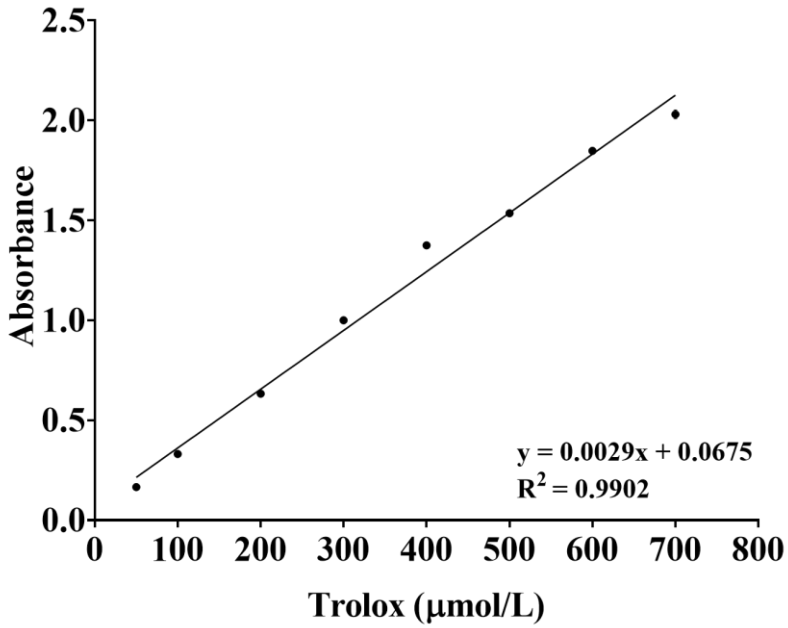
APPENDIX A.7. Trolox Standard for DPPH Radical Scavenging Assay



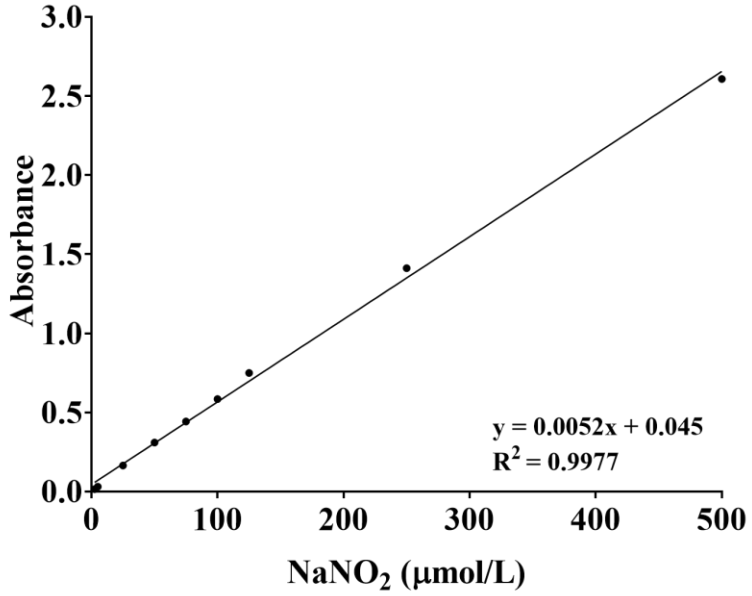
APPENDIX A.8. Trolox Standard for Ferric Reducing Antioxidant Power (FRAP) Assay



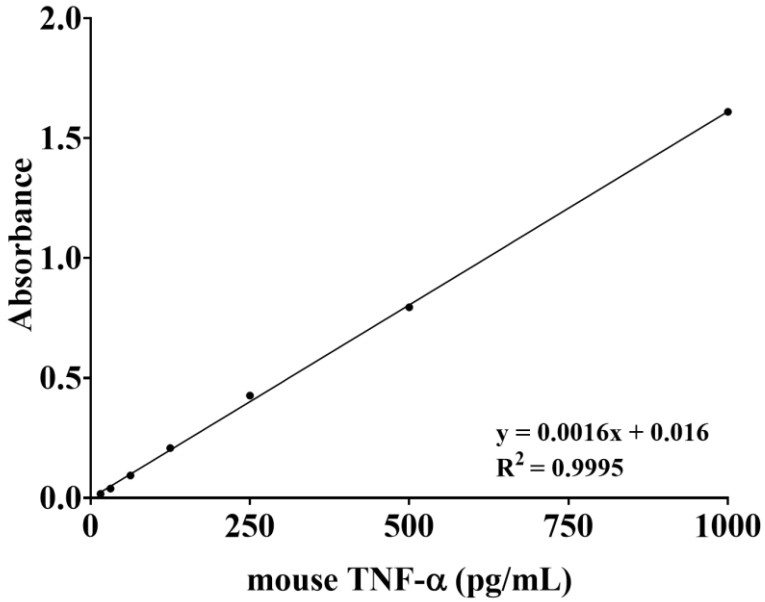
APPENDIX A.9. Ascorbic Acid Standard for Ferric Reducing Antioxidant Power (FRAP) Assay



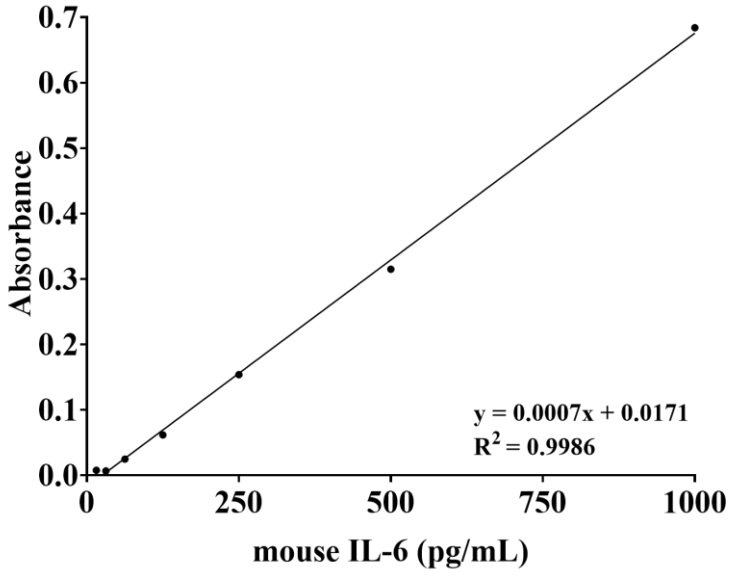
APPENDIX A.10. Nitric Oxide Standard for Griess Assay



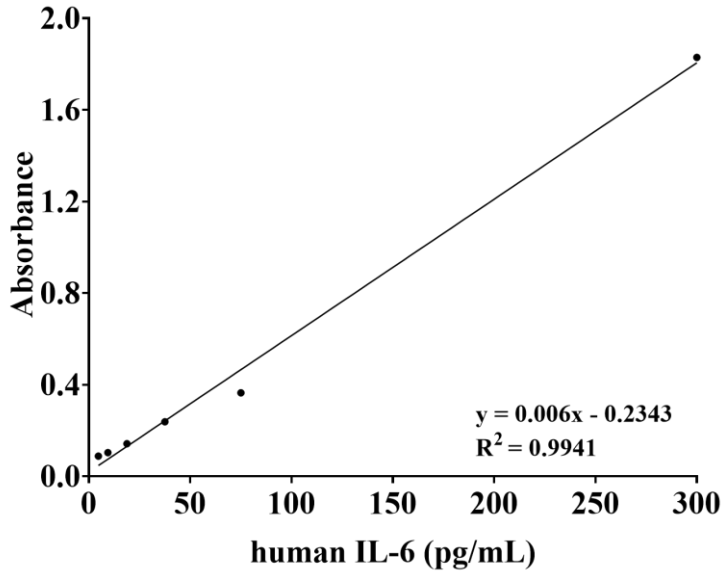
APPENDIX A.11. Mouse Tumor Necrosis Factor Alpha (TNF-α) Standard for Enzyme-Linked Immunosorbent Assay (ELISA)



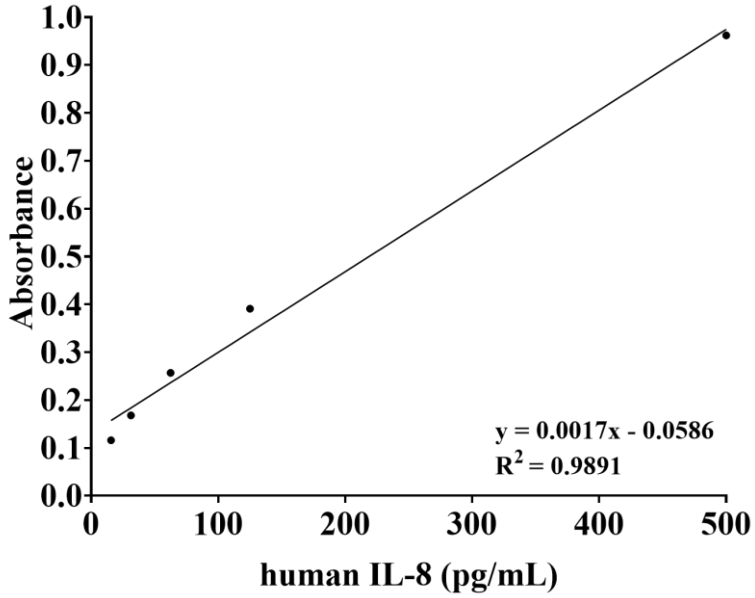
APPENDIX A.12. Mouse Interleukin 6 (IL-6) Standard for Enzyme-Linked Immunosorbent Assay (ELISA)



APPENDIX A.13. Human Interleukin 6 (IL-6) Standard for Enzyme-Linked Immunosorbent Assay (ELISA)



APPENDIX A.14. Human Interleukin 8 (IL-8) Standard for Enzyme-Linked Immunosorbent Assay (ELISA)



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Ozel-Tasci, C., Pilatin, G., Edeer, O., Gulec, S. (2020). *In vitro* assessment of food-derived-glucose bioaccessibility and bioavailability in bicameral cell culture system. *Turkish Journal of Food Biochemistry*, 45: 631-637.

Ozel, C., El, S.N. (2016). Release of total carotenoids and β-carotene from pumpkin products during in vitro digestion. 1st International Conference on Food Bioactives and Health.

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