RESEARCH OF PHYTOCHEMICAL AMPK ACTIVATORS AGAINST OBESITY AND DEVELOPMENT OF DIETARY SUPPLEMENTS

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RESEARCH OF PHYTOCHEMICAL AMPK ACTIVATORS AGAINST OBESITY AND DEVELOPMENT OF DIETARY SUPPLEMENTS

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ABSTRACT

RESEARCH OF PHYTOCHEMICAL AMPK ACTIVATORS AGAINST OBESITY AND DEVELOPMENT OF DIETARY SUPPLEMENTS

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A fit and healthy life style has been more and more accepted throughout the world. In that sense, an increasing interest on phytochemicals has been recognized as developing dietary supplements against obesity. Many related products in the markets were hazardous and their uses have caused irremediable results, even in death. For this very reason, this study was intended scientifically to develop a true dietary supplement for obesity.

In the energy metabolism, AMP-activated protein kinase (AMPK) has a significant role over the pathway enzymes. AMPK has also been implicated in the central regulation of food intake and energy expenditure. Thus, appropriate

phytochemicals as the activators of AMPK might make the recovery possible from obesity.

In this study, the selected plants were *Citrus limon* (as peel and juice), *Berberis integerrima* and *Taraxacum bessarabicum* which have been used in folk medicine for appetite suppression. Extracted and fractionated plants examined for their phenolic contents as well as for their radical scavenging capacities through the methods of DPPH and ABTS.

The extracts showing high radical scavenging capacity were carried out for the treatments on adipocyte-like (differentiated 3T3-L1) cells. The AMPK activation in the differentiated 3T3-L1 cells was monitored via phosphorylation assay using fluorescence detected ELISA. Moreover, the AMPK protein expression was observed by qRT-PCR.

The ethyl acetate fractionated extracts of *C. limon* peel, *B. integerrima* and *T. bessarabicum* were found to be effective AMPK activators at concentrations of as low as 10 μg/mL. Consequently, they can be considered as suitable extracts for the development of fat reducing dietary supplements.

Keywords: Obesity, AMPK activity, antioxidant activity, dietary supplements

ÖZ

OBEZİTEYE KARŞI FİTOKİMYASAL AMPK AKTİVATÖRLERİNİN ARAŞTIRILMASI VE GIDA TAKVİYESİ GELİŞTİRİLMESİ

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Formda ve sağlıklı bir yaşam tarzı dünyada giderek daha fazla kabul görmüştür. Bu bağlamda, fitokimyasallar üzerine artan ilgiyle, obeziteye karşı diyet takviyeleri geliştirilmesi hız kazanmıştır. Piyasalardaki pek çok tehlikeli ürünün kullanımı, geri döndürülemez sonuçlara hatta ölümlere sebep olmuştur. Bu nedenle, bu çalışma obezite için bilimsel olarak kanıtlanmış gerçek bir besin takviyesi geliştirmeyi amaçladı.

AMP-aktive protein kinaz (AMPK) Enerji metabolizmasında bulunan yolak enzimleri üzerinden önemli bir role sahiptir. AMPK, gıda alımı ve enerji harcamalarının düzenlenmesiyle de ilişkilendirilmiştir. Böylece, uygun

fitokimyasallar AMPK aktivatörü olarak obeziteden kurtulmayı mümkün kılabilir.

Bu çalışmada, seçilen bitkiler *Citrus limon* (kabuk ve meyve suyu olarak), *Berberis integerrima* ve *Taraxacum bessarabicum* halk arasında iştah kesme amaçlı kullanılmaktadır. Özütlenen ve fraksiyonarına ayrılan bu bitkiler, fenolik içerikleri ve ayrıca DPPH ve ABTS yöntemleri ile radikal süpürme kapasiteleri açısından incelendi. Ardından, yüksek radikal süpürme kapasitesi gösteren özütler, adiposit benzeri hücreler (farklılaştırılmış 3T3-L1) üzerinde yapılan muamelelerde kullanıldı. Farklılaştırılmış 3T3-L1 hücrelerindeki AMPK aktivasyonu, floresan ELISA kullanılarak fosforilasyon deneyleri ile izlendi. Buna ek olarak, AMPK protein ekspresyonu nicelikli gerçek zamanlı PCR ile gözlendi.

Etil asetat fraksiyonlarına ayrılmış *C. limon* peel, *B. integerrima* and *T. bessarabicum* özütlerinin, 10 µg/mL'ye kadar düşük konsantrasyonlarda etkili AMPK aktivatörleri olduğu bulunmuştur. Sonuç olarak, bu özütler zayıflatıcı diyet takviyeleri geliştirilmesi için uygun olarak düşünülebilir.

Anahtar kelimeler: Obezite, AMPK aktivitesi, antioksidan aktivite, gıda takviyesi

To my family

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LIST OF ABBREVIATIONS

mg	Milligram
mL	Milliliter
µL	Microliter
nm	Nanometer
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
IC_{50}	Inhibitory Concentration
RSA	Radical Scavenging Activity
TP	Total Phenol
TF	Total Flavonoid
GAE	Gallic Acid Equivalent
CE	Catechin Equivalent
DMSO	Dimethyl Sulfoxide
SD	Standard Deviation
TEAC	Trolox Equivalent Antioxidant Capacity
XTT	2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide innersalt
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
PBS	Phosphate buffered saline
cDNA	Complementary DNA
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
Ct	Threshold Cycle
mRNA	Messenger RNA

CHAPTER 1

INTRODUCTION

1.1 Obesity

Obesity is defined as excessive accumulation of adipose tissue. There are several ways to determine the amount of fat texture. The most common one is calculation of Body Mass Index (BMI) involving height and weight. BMI is calculated by dividing a person's body weight in kilograms by his/her height in meters squared. As displayed in **Figure 1.1,** a person is considered as underweight when BMI is below 18.5; as normal weight when it is between 18.5-24.9; as overweight between 24.5-29.9; as obese greater than 30; and is considered as morbid obese greater than 40.

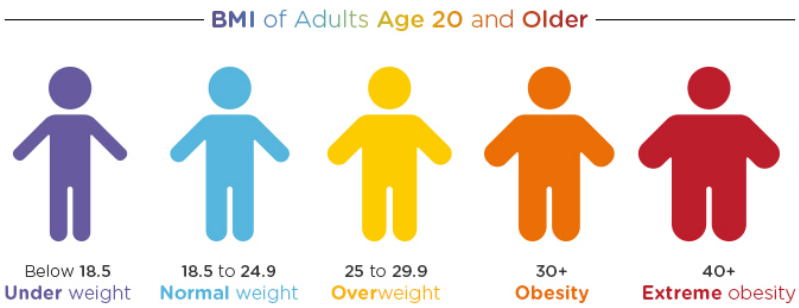


Figure 1.1: Body Mass Index of adults age 20 and older (www.med4u.co.uk)

Obesity, especially the morbid obesity, leading to the metabolic disorders like cardiovascular diseases and diabetes, affects more than one million people each year. Obesity, being one of the leading causing of death will increase over the years, unless any action is taken against it. According to the study published by World Health Organization (WHO) one third of the world's population is obese. When a person's Body Mass Index is 30 or more, he or she is considered as obese, and 37% of men and 38% of women are stated as overweight.

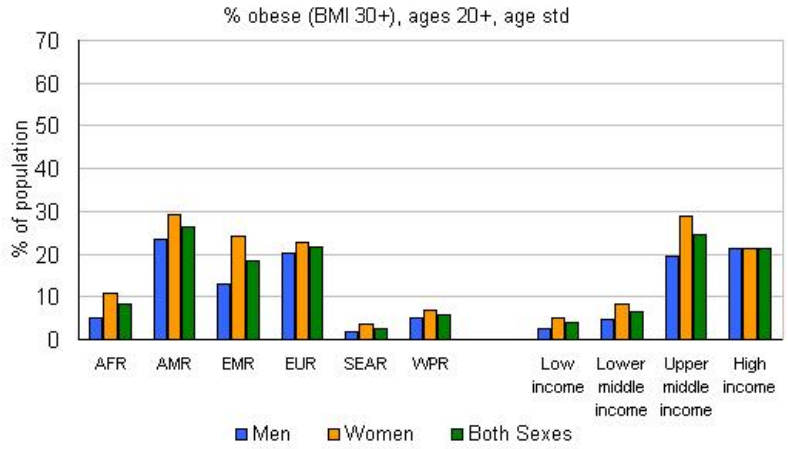


Figure 1.2: The prevalence of obesity in the regions of World Health Organization (www.who.int)

According to WHO, the prevalence of overweight and obesity is the highest in America (62% for overweight in both sexes, and 26% for obesity) and lowest in South East Asia (14% overweight in both sexes and 3% for obesity) as shown in **Figure 1.2**. In Eastern Mediterranean, Europe and in America over 50% of female population is overweight and almost half of those are considered as obese (24% in the Eastern Mediterranean, 23% in Europe, 29% in the Americas). In the WHO regions, women seem to be more prone to obesity than men. In Africa, Eastern Mediterranean and South East Asia, the prevalence of obesity in women is twice of men.

In 1990, presence of obesity among adults was only 18.6 %, a decade later however, it was 21.9 %, which was a 17.7 % increase. The world-wide statistics was also true for Turkey, that the men were more overweight while obesity was more common among women (Yumuk, 2005).

According to data given by Turkish Society of Cardiology (TSC) in the study of TEKHARF, 3681 subjects with BMI of 30 kg/m^2 were examined. Over 30 years of age, one third of the men (25.2%) and roughly half of the women (44.2%) were considered as obese. When middle-age (31-49 age) and elder groups (50 years and older) were taken into account, the obesity prevalence in women significantly increased by age, however prevalence in men did not show any difference. It was also mentioned that, in 1990, obesity prevalence was 12.5 % among the groups of same age, was 2 fold higher in men compared to women. TOHTA study had been carried out between the years of 1999- 2000, with the involvement of 23.888 adults. Even among the young women of 20 years of age prevalence of obesity was 35.4% which was 1.8 fold higher than men. According to TURDEP study completed with 24.788 people, over 20 years old; obesity prevalence (BMI 30 kg/m2) was found as 29.9% in women and 12.9% in men. The high prevalence of obesity in women showed that they may have serious problems such as cardiovascular diseases and type 2-diabetes.

1.2 AMP-activated Protein Kinase

AMP-activated protein kinase (AMPK) has a significant role over the enzymes taking place in the pathway of energy metabolism. AMPK is activated by metabolic stress either by inhibiting ATP synthesis or by accelerating ATP consumption.

AMPK activity related shifts in lipid and glucose metabolisms results in the increase of obesity dependent pathogenesis, such as type 2 diabetes and other metabolic symptoms.

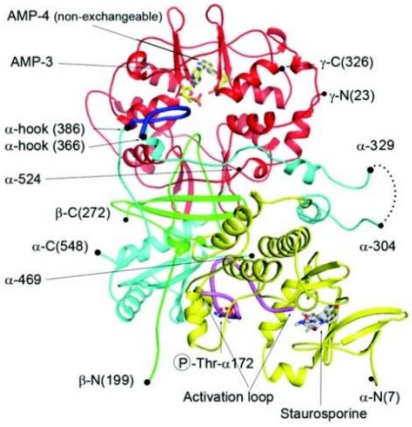


Figure 1.3: Structure of AMP-activated protein kinase (AMPK) (Lage, 2008)

In the presence of metabolic stress, cellular AMP/ATP ratio shows an increase that triggers AMPK activity by the phosphorylation of Thr172 in the catalytic subunit (Hardie, 2003; Carling, 2004; Kahn, 2005) as shown in the structure of AMPK in **Figure 1.3.**

In adipose tissue, the alpha1 catalytic subunit of AMPK is the major isoform responsible for AMPK activity that is expressed predominantly (Lihn, 2004; Daval, 2005).

AMP binding to alpha-subunit of AMPK results in a 2 to 5-fold activity increase in the AMPK. On the other hand binding of AMP to gamma-subunit induces a conformational change preventing de-phosphorylation of Thr-172 on alpha-subunit.

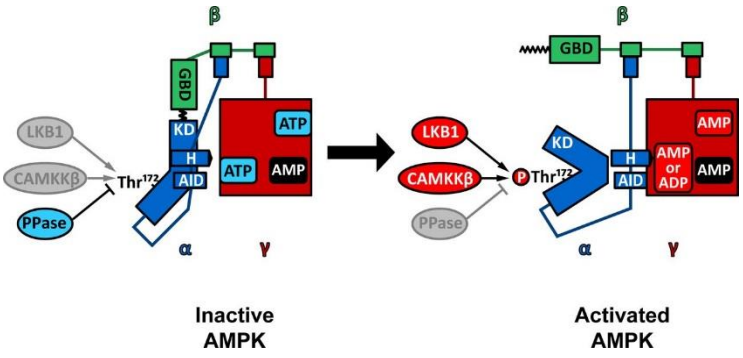


Figure 1.4: Representation of inactive and activated AMPK (Zaha, 2012)

As mentioned before, AMPK is activated by phosphorylation of α-catalytic subunit at Thr172 by Liver Kinase B1 (LKB1) or Calcium/Calmodulin-dependent Protein Kinase 2 (CaMKKβ). Increasing intracellular concentration of Ca2+ triggers CaMKKβ-mediated AMPK activation independent of the adenine nucleotide ratios as seen in **Figure 1.4** above.

Among the prescribed antidiabetic drugs metformin and rosiglitazone (thiazolidinedione), increase the concentrations of AMP/ATP or ADP/ATP that triggers the activation of AMPK. The plant-derived compounds such as resveratrol, galgeine and berberine also activate AMPK by this mechanism (Bijland, 2013). Upon activation, AMPK provides energy balance in cellular pathways through inhibition/activation of the pathways, such as fatty acid synthesis, cholesterol synthesis, gluconeogenesis, etc. as shown in **Figure 1.5**.

Subsequently the activation of AMPK is controlled by the phosphorylation of the metabolic key enzymes, where the longer term activation can be achieved through gene expression (Hardie, 2007; Hardie, 2008).

AMPK is associated with the central regulation of food intake and energy cost in response to the signaling of hormones such as ghrelin, leptin, and adiponectin (Dzamko, 2009).

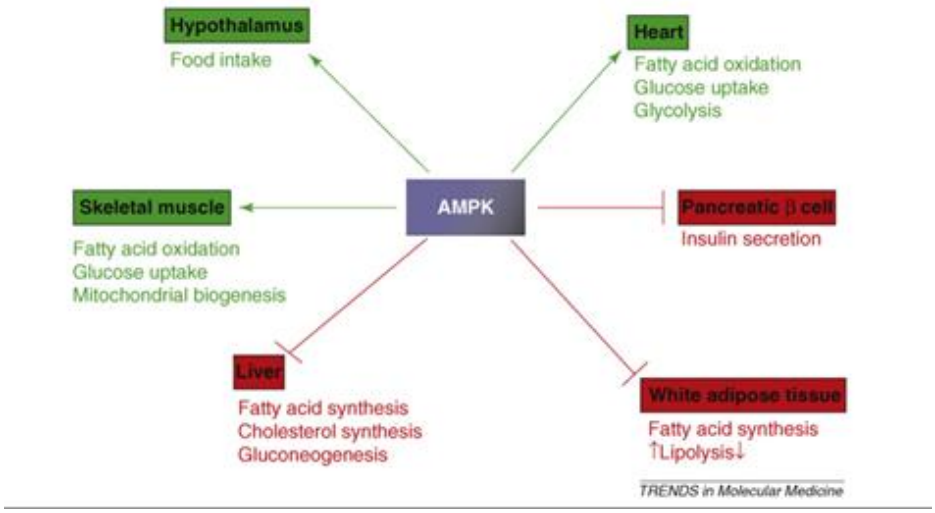


Figure 1.5: Roles of AMP-activated protein kinase (AMPK) in the control of energy metabolism (Lage, 2008)

AMPK is a major regulatory enzyme in the pathway of lipid biosynthesis by activation and inactivation of the key enzymes such as acetyl-CoA carboxylase (ACC) (Hardie, 1997).

Recently, it was pointed out that AMPK has an extensive role in fatty acid oxidation of metabolic regulation (Winder, 1999) and in muscle glucose uptake (Hayashi, 1998; Merrill, 1997; Goodyear, 2000). AMPK is also employed in the expression of cAMP-stimulated genes in gluconeogenesis (Lochhead, 2000), as well as in the expression of glucose-stimulated genes in relation to hepatic lipogenesis (Foretz et al., 1998).

Continuous activation of AMPK may mimic the effects of extensive exercise through stimulation of muscle hexokinase and glucose transporters (Glut4) expression (Holmes, 1999).

Upon depletion of the cellular ATP provokes stressful conditions of hypoxia and low glucose level prompts AMPK activation. In the AMPK signal pathway, there are enzymes phosphorylating AMPK through transcriptional control of the metabolism. As an example, increasing c-AMP concentration in cell turns on PKA activation which is followed by the trigger of LKB1, and subsequently AMPK activation takes place as displayed in **Figure 1.6**.

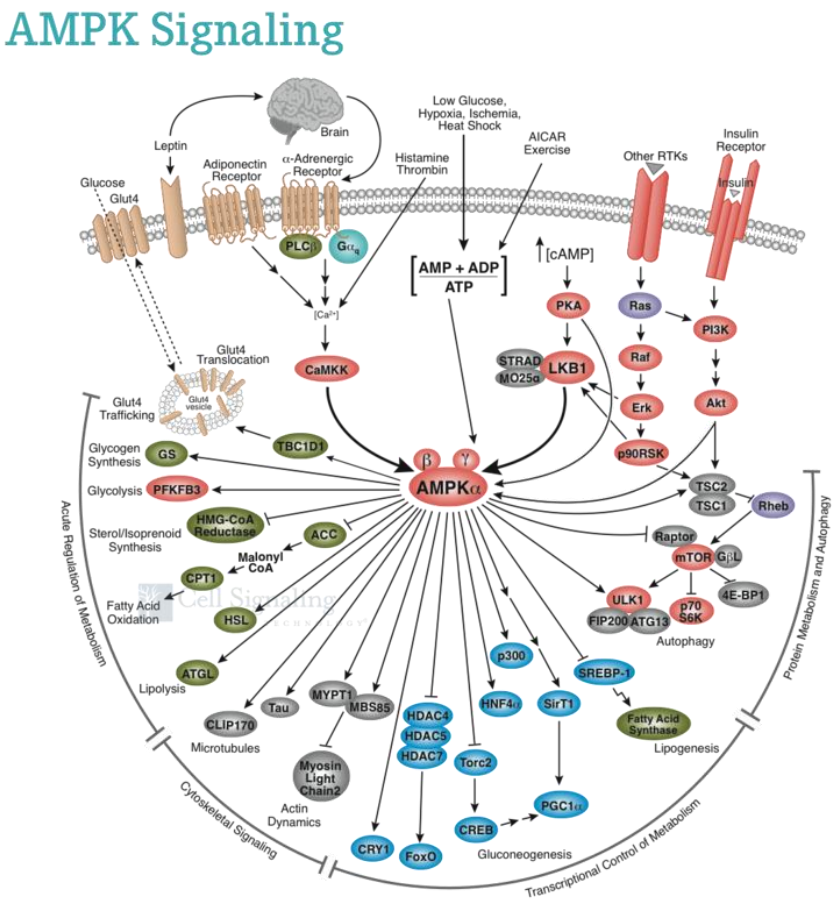


Figure 1.6: AMP Kinase signaling pathway (Cell Signaling Technology, 2014)

Recently, AMPK has been identified as a potential target in the treatment of cancer. In the cell growth and proliferation mechanisms AMPK can serve as an energy controller. The activators of AMPK were anticipated as the new approaches in the therapy for the conditions of cancer and metabolic syndrome the two of which are considered as the globally most widespread diseases (Fogarty, 2010).

1.3 AMPK as a Drug Target

In the last couple of decades, AMPK seemed to be an inspiring target for the development of novel drugs, for the cure of metabolic syndrome as obesity-related cardiovascular diseases and type-2 diabetes. More recently, AMPK's role in the regulation of cell growth and proliferation has been stated and it may also lead as a target in cancer therapy (Wang, 2009).

Among the drugs used for metabolic syndrome, metformin was one of the most commonly drugs for type-2 diabetes, since 1960s. Metformin is used as a medication for hyperglycemia to avoid weight gain and excessive insulin secretion in the patients with metabolic syndrome (Wiernsperger, 1999). High concentrations of metformin (10 mM) has been found to reduce total ATP levels in cells (Cusi, 1998), and it was also stated that slight changes in the ratio of free ATP/ADP can take place with concentrations of metformin while these concentration are not enough to suppress total ATP, however enough to inhibit gluconeogenesis (Owen et al., 2000). In another report it was pointed out the metformin treatment in the concentrations of 500–2,000 μM; did not affect total ATP concentrations in rat hepatocytes (Zhou, 2001). Although, the role of AMPK in insulin resistance mechanism was defined as the inhibition of pancreatic insulin secretion from the β-cells, however its role in lipolysis is still not well-known (Lage, 2008).

1.4 Phytochemicals as AMPK Activators

Phenolic constituents of plant material in their defense systems having electron scavenging or donation capability are considered as antioxidants. Antioxidant molecules in fruits, vegetables and grains are important in the protection of oxidative stress related diseases (Yu, 2002).

The family of phenolics contains nearly 8000 compounds identified (Bravo, 1998). Flavonoids are the most important part of the phenolic compounds commonly found in vegetables, fruits, nuts and beverages such as coffee, tea, and red wine (Hollman, 1997; Heim, 2002; Clifford, 2000).

Flavonoids contain two aromatic rings with at least one hydroxyl group and 15 carbon atoms are among the water soluble members of phenolic compounds as shown in **Figure 1.7**. Flavonoids can also be sub-classified into six groups which are flavanol, flavanone, flavone, flavonol, anthocyanins and isoflavonoids (Tura, 2002; Bravo, 1998).

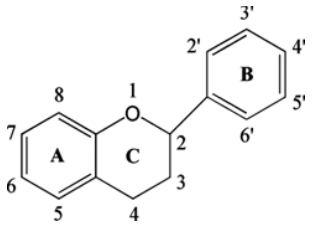


Figure 1.7: Skeletal structure of flavonoids

Plant polyphenols are important for their protection against many diseases related to metabolic disorders. They are part of an increasing interest in human

health for their phytochemicals. Those phytochemicals are effective on metabolic enzymes among them AMPK has an important role in lipid metabolism.

There are lots of phytochemicals which have been identified as the novel activators of AMPK and one of them is berberine**,** a plant alkaloid used in traditional Chinese medicine. Berberine was reported in literature for improving insulin sensitivity and as well as increasing AMPK activity in muscle and hepatic cell lines, and 3T3-L1 cell line (Kim, 2007; Lee, 2006; Yin, 2007).

Berberine itself seems to control lipid metabolism by interfering in AMPK activity. AMP-activated protein kinase (AMPK) has an eminent role in energy balance in the entire system. In the treatment with berberine liver weight, hepatic and plasma triglyceride, and cholesterol contents were observed to be reduced. Additionally, intra- ventricular administration of berberine declined the level of malonyl-CoA, at the same time in skeletal muscle increased the expression of fatty acid oxidation genes (Kim, 2009). Another phytochemical example is resveratrol. It is a polyphenol found in red wine and also shown to activate AMPK in cultured cells (Baur, 2006; Dasgupta, 2007).

There have been many natural products reported to stimulate AMPK activation. Among those, quercetin (Suchankova, 2009; Ahn, 2008), curcumin (Fujiwara, 2008) isoginkgetin (Liu, 2007) ginsenoside (Hwang, 2009) and triterpenoids (Tanabe, 1975) can be counted. It is challenging to realize how so many drugs and phytochemicals of unlike structures could possibly function as an AMPK activator.

In this study, AMPK activators are searched among plants for developing natural dietary supplements with the anticipation of preventing obesity in near future.

1.5 Plants in This Study

Natural products of plant origin are used for many purposes including diet, personal care and cosmetics. They have important effect on human health with their anti-oxidant, anti-cancer, anti-inflammatory, anti-microbial, and anti-obesity properties. Here, some plants which were used by Turkish people to lose weight and to suppress the appetite were selected for this thesis study.

Citrus limon (L.) Burm.f., from Rutaceae family, is a small leafless tree species native to Asia. The lemon juice is primarily used for its fruit juice throughout the world for and is believed to be effective on weight loss.

Berberis integerrima Bunge, from Berberidaceae family; is an important medicinal plant with its yellow wood, yellow flowers and red fruits. It is grown in the eastern regions of Turkey and is traditionally used for its antioxidant, anti-inflammatory, hypoglycemic properties .

Taraxacum bessarabicum (Hornem.) Hand.-Mazz. subsp. *bessarabicum*, commonly known as dandelion, belongs to Asteracea family. The dandelion is found all over the continents and used for infections, bile and liver problems. It is also used as a diuretic agent. Native Turkish people, consume its tea for weight loss, although there is no scientific evidence about it.

These three plants have been used in the treatment of different diseases for centuries. In this study *C. limon* peel and juice, *B. integerrima* and *T. bessarabicum* (**Figure 2.1**) extracts, which are used in Turkish folk medicine for apetite suppression and weight control, were evaluated for their bioactive potentials on AMPK enzyme as an activator.

1.6 Dietary Supplements

Supplemental nutrient intake is very beneficial in times of deficiency (Silver, 2009). Dietary supplements are products designed to increase daily nutrient intake, usually vitamins and minerals.

Dietary supplements are not regulated as strictly as drugs in Turkey, as well as in the whole world. The Food and Drug Administration (FDA) maintains a list of hazardous products that are marketed as dietary supplements. Manufacturers do have to follow some rules regarding labeling and about the supplements. Most dietary supplements are safe as long as you follow the label instructions, but in some large doses of some food species there may be some strong biological effects on the body. Therefore, it is really important to produce dietary supplements based on scientific studies.

1.7 3T3-L1 Cell Line

From the studies in last 25 years, two in vitro models were used for understanding the mechanisms in adipocyte proliferation, differentiation and adipokine secretion. First one is pre-adipocyte cell lines that have the ability of differentiation into adipocytes such as 3T3-L1. Second one is multi-potent stem cells which are able to differentiate into different cell lines such as adipose, muscle and bone.

3T3-L1 is a cell line which is derived from mouse *mus-musculus*. Since 3T3-L1 cells have a fibroblast-like morphology, they have the ability of differentiation

into adipocyte-like phenotype if the conditions were prepared they can be used in obesity research (Green, 1975).

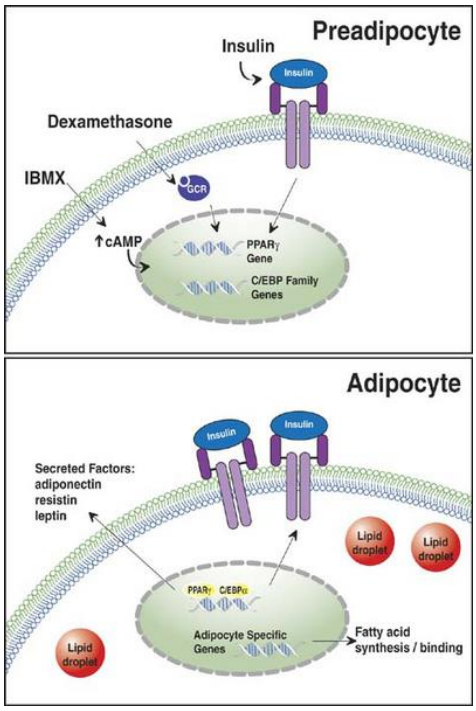


Figure 1.8: Representation of the differentiation of pre-adipocytes into adipocytes (MerckMillipore.com)

As it was shown in **Figure 1.8**, pre-adipocyte differentiation is stimulated by endocrine and autocrine factors. These factors induce the synthesis and activation of transcription factors in adipogenesis. Upon treatment of 3T3-L1 cells with the induction chemicals of methylisobutylxanthine, dexamethasone, and insulin (AD) containing media, the expression of transcripton factor C/EBPβ and C/EBPδ is rapidly induced.

Following 2-3 days, with the expression of C/EBPβ/δ 3T3-L1 preadipocytes undergo many cellular division process called mitotic clonal expansion (MCE). Then, the expression of C/EBPα and peroxisome proliferator-activated receptor gamma (PPARγ) induced to cease MCE and the expression of genes in triglyceride metabolism leads to formation of lipid droplets indicating a differentiated mature adipocyte.

Adipocyte is the fat cell, which is specialized in fat synthesis and storage. In response to hormonal stimulus, adipocytes store energy with the accumulation of lipids. Under the microscope, they seem to be swollen and full with lipid droplets. When mesenchymal adipocyte precursor cells are cultured in a differentiation medium that is composed of insulin, dexamethasone and IBMX, expression of adipocyte markers was shown to be increased (Ringold, 1988; Flier, 1995; Cornelius, 1994; Spiegelman, 1993).

1.8 Scope of Thesis

It becomes impossible for the morbid obesity patients to lose weight by the help of exercise, due to the excess weight and the resulting cardiovascular deficiencies. In such a vicious cycle, it is inevitable that the obesity becomes a permanent metabolic disease, due to the lack of exercise. In this case, it is essential to develop methods for increasing metabolic fat burning processes.

The interest on phytochemical compounds has been increased especially as the supplements for prevention of obesity. It is aimed to obtain fat burning dietary supplements using potent phytochemicals as the AMPK activators, based on the scientific results completed with in vitro toxicity tests.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant Material

The plant materials which were shown in **Figure 2.1**, were identified and collected by Associate Professor Dr. Fevzi Ozgokce and his research group, Deparment of Biology, Yüzüncü Yil University.

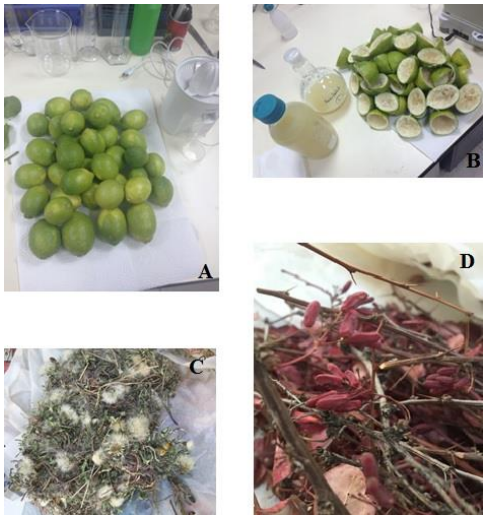


Figure 2.1: The samples which were used throughout the study;

A) *C. limon* peel B) *C. limon* juice C) *T. bessarabicum* D) *B. integerrima.*

Information about the plants was given in the **Table 2.1**. The aerial parts of *T. bessarabicum* and *B. integerrima* were used. *C. limon* samples were used after it is peeled and juice was obtained.

Table 2.1: Plants which were used throughout the experiments

Species	Family	Location	Collection Time/ Voucher Number
Citrus limon (L.) Burm.f.	*Rutaceae*	Antalya: Konyaalti, Kurma District, Bogacay Street, Lemon Garden	15.07.2014 F14134
Berberis integerrima Bunge	*Berberidaceae*	Van: Yuzuncu Yıl University, Science Faculty, Back of Biology-Chemistry building, Inner gardens	10.10.2014 F14135
Taraxacum bessarabicum (Hornem.) Hand.-Mazz. subsp. *bessarabicum*	*Asteraceae*	Van: Catak, Sirmali Village, East side of Ardıçlı Arable Field, steppe.	26.06.2015 F14136

2.1.2 3T3-L1 Cell Line

CL-173™ (3T3-L1) cell line, which constitutes the basic properties of a mus musculus, was purchased from ATCC® (American Type Culture Collection).

2.1.3 Chemicals and Other Materials

Preparative chromatography grade methanol used in the preparation of extract was purchased from Merck (Darmstadt, Germany). For the fractionation process, hexane, chloroform and ethyl acetate were purchased from Merck (Darmstadt, Germany) and magnesium sulfate was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Water was purified by Millipore Simplicity 185 system to ultra-pure water (Millipore, Bedford, MA, USA). Disposable syringe filters in pore size of 0.45 μm and 0.22 μm were purchased from Millipore Corporation (Bedford, MA USA) and syringe filter (0.20 μm Minisart RC 4), from Sartorius (Gottingen, AG Germany).

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate (Na_2CO_3) were purchased from from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Folin Ciocalteu's phenol reagent, aluminium chloride ($AlCl_3$) were purchased from Merck (Darmstadt, Germany). All of the reference compounds such as gallic acid, ascorbic acid, esculin, esculetin, catechin, caffeic acid, epicatechin, p-coumaric acid, scopoletin, ellagic acid, quercetin, apigenin, hesperetin, rutin, trolox were all purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA).

Dimethyl sulphoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM), ATCC® 30-2002; and Bovine Calf Serum, ATCC® 30-2030 were purchased from ATCC®. Fetal Bovine Serum (heat-inactivated) were purchased from Biochrom Ltd. (Cambridge, UK). Dulbecco's Phosphate Buffered Saline (PBS) was purchased from Biological Industries (Haemek, Israel). Trypan blue stain 0.4 % and Counting Slides, used with Juli™ Br were purchased from NanoEnTek (Guro-gu, Seoul, Korea).

XTT Kit for cytotoxicity assays was purchased from Biological Industries (Israel). For RNA Isolation, Life Science innuPREP RNA mini kit (AnalytikJena) and for c-DNA synthesis, Transcriptor High Fidelity cDNA synthesis kit (Roche, Germany) were used.

Trypsin-EDTA Solution (%0.25) and antibiotic-antimycotic solution (100x) were purchased from Sigma (USA). DNase/RNase free distilled water was purchased from Gibco (Life Technologies).

For cell differentiation, Insulin (bovine) and Methylisobutylxanthine (IBMX) were purchased from Sigma® and Dexamethasone was purchased from G Biosciences®. Eppendorph Easypet micropipette and LP Italiana Spa 5,10,25 mL steril pyrogen free plastic pipettes were used in cell culture experiments. Sterile plastic falcon tubes were purchased from Orange Scientific and ISOLAB. DNase/RNase free, 2 mL of Cryo.s™ were purchased from Greiner Bio-one.

T25 and T75 cell culture flasks and 96-well plates were purchased from Cellstar. Corning® clear bottom, black polystyrene 96-well plates were purchased from Sigma. All the reagents, chemicals and materials used in cell culture studies were cell culture grade.

2.1.4 Instruments

Bandelin Sonorex ultrasonic bath; Optic Ivymen System incubator; Rotary Evaporator (Heidolph Laborata 4000 and Hei-VAP Advantage); Waring Blender 32BL80 (New Hartford, CT, USA), Lyophilizator (Heto-Holten Model Maxi-Dry Lyo in the Central Lab of Biological Sciences Department , METU) were used in preparing plant samples. Airstream Class II Biohazard Safety Cabinet (ESCO, Thailand),) Juli-Br and FL Station Live Cell Movie Analyzer (NanoEnTek Inc.), Nüve EC160 CO_2 incubator and Nüve NF615 Centrifuge were used in cell culture assays.

For 96- well plate measurements Multiskan™ GO Microplate Spectrophotometer (ThermoFisher Scientific, USA) was used, in Biological Sciences Department, METU.

Microlite RF, Thermo Centrifuge (Thermo Electron Corporation) in Prof. Dr. Orhan Adalı's Laboratory, was used for centrifuge.

Mettler TOLEDO, MPC227 pH/conductivity meter, WiseStir® MSH-20A (Wisd Laboratory Instruments) Magnetic stirrer, Memmert WB14 waterbath, Precisa XB 220A and Sartorius BL 1500 were used.

FLUOstar OPTIMA ELISA reader (BMG Labtech) was used in cytotoxicity and enzyme assays.

Conventional thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems), AlphaSpec Nanodrop (Alpha Innotech), and LightCycler 480 qRT-PCR (Roche) were used in Central Laboratory of Biological Sciences, Middle East Technical University. Primers were synthesized in Oligomer Biotechnology.

2.2 METHODS

2.2.1 Preparation of the Samples

After the extracts of the plants were obtained, fractionation was carried out as described below.

2.2.1.1 Extraction Procedure

A sufficient quantity of dry plants (quantities and yields were given in **Table 3.2**) were grinded to small particle size with a commercial blender at a high speed for at least 5 minutes and stored at room temperature, in a dry and dark place until use. To obtain the plant extract, *T. bessarabicum* and *B. integerrima*; peel of *C. limon* were incubated with methanol in the ratio of 1:6 in oval-rotating incubator by rotating at 180 rpm at 25 °, for 24 hours. Then, mixtures were filtered through filter papers. This procedure was applied according to Coruh et al. (2007) and it was repeated three times. Collected filtrates were completely dried by the help of a rotary evaporator under vacuum at 40 °C. *C. limon* juice was lyophilized immediately. Crude extract samples were weighed for the calculation of the yields.

2.2.1.2 Fractionation Procedure

Varying amounts of crude extracts were dissolved in methanol-water mixture of total 500 mL (70:30, v/v) to ensure solubility of hydrophilic components. Methanol-water mixture was poured into a separation funnel with the addition of the same volume of hexane in a separator funnel. The mixture was shaken

vigorously and kept until the organic phases were separated. When aqueous and organic phase separation obtained by fractionation, separated organic phase was drained into the beakers and evaporated. The procedure was carried out with other organic solvents in an increasing polarity; with chloroform and ethyl acetate. At the end the remaining aqueous phase was collected and lyophilized. All of the organic phases collected from fractionation method were evaporated to dryness and they were stored at +4 °C for the experiments. Each of these separation processes were repeated 3 times until organic phase was completely transparent. The fractionation method described by Naczk and Shahidi (2004) was used to obtain the fractions of the plant crude extract. Extraction and fractionation procedure for all four plant samples are summarized in **Figure 2.2.** The yield of each fraction was determined in terms of (w/w) % per g crude extract.

2.2.2 Determination of Antioxidant Capacity

All assays were done in µL scale with 96 well plates to screen antioxidant activities and to determine total phenolic and total flavonoid contents.

2.2.2.1 Free Radical Scavenging Activity by DPPH

A modified protocol of DPPH (2,2-diphenyl-1-picrylhydrazyl radical) procedure was utilized to determine DPPH radical scavenging capacity (Blois, 1958) with some modifications. Flat bottom 96-well plates were used to measure the radical scavenging capacity of the plant extracts which were prepared in methanol, as serial dilutions between 1.56-50.00 mg/mL concentrations, and ranging from 0.10- 3.33 mg/mL as final concentrations in wells.

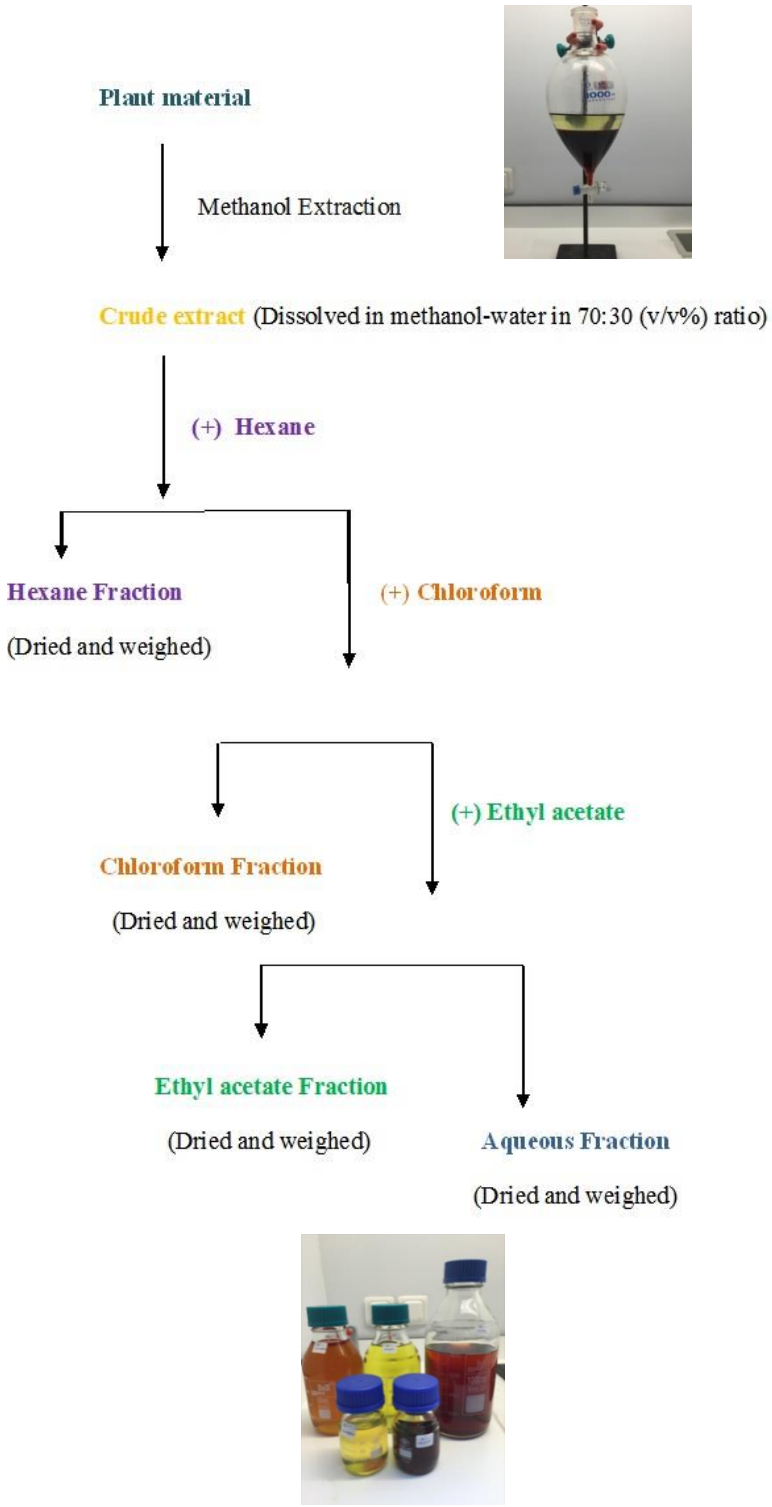


Figure 2.2: General description of the fractionation procedure

Basically; DPPH solution was prepared by dissolving 20 mg of DPPH radical in 400 mL absolute ethanol to make the final concentration 0.05 mg/mL (0.127 mM). 10 µL of each extracts were mixed with 140 µL DPPH solution in 96-well plate.

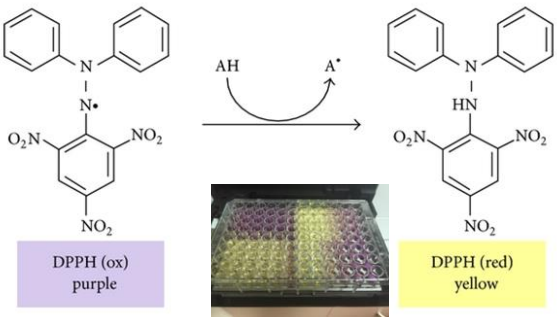


Figure 2.3: Principle of DPPH radical scavenging activity assay (Teixeira, 2013)

Due to depletion of DPPH radical; radical scavenging capacities of the crude extracts and their fractions, along with the standard reference compound, were determined by observing the reduction in the absorbance at 517 nm, 30 minutes after the reaction **(Figure 2.3)**. The results were expressed as the radical scavenging activity (RSA) in percent and calculated as:

Radical Scavenging Activity (%) = 100 x [(Absorbance of Sample with DPPH) - (Absorbance of Sample Blank)]/[(Absorbance of DPPH)-(Absorbance of Solvent)]

The half maximal inhibitory concentration (IC_{50}) is the measure of the efficiency of a substance in inhibiting a biochemical response. It was calculated as the concentration of enough substance to reduce the % 50 DPPH radical, followed by a color change. All experiments were carried out with 6 different concentrations in quadruplicates and repeated as two different experiments.

2.2.2.2 Free Radical Scavenging Activity by ABTS

ABTS method was used for the determination of antioxidant capacity. Basically this method is based on the ability of antioxidant compounds to scavenge free radicals as equivalent of trolox standard (Rice-Evans, 1997).

Figure 2.4: Reaction between $ABTS^{\bullet+}$ radical and antioxidant (Apak, 2007)

According to the reaction between ABTS and potassium persulfate ($K_2S_2O_8$), ABTS radical is generated and production of a green ABTS chromospheres are observed as illustrated in **Figure 2.4.** With the addition of antioxidant samples the radical is reduced and the absorbance is read at 734 nm (0.7±0.02 absorbance unit). After 6 minutes, reaction stops and the absorbance become stable.

The ABTS radical cation ($ABTS^{\bullet+}$) produced by preparing a solution by mixing 7 mM of ABTS and 2.45 mM of potassium persulfate. Then this mixture was kept in dark, for 16 h, at room temperature in a shaking incubator.
The solution was diluted by adding 100 mL of 96 % ethanol to make the final absorbance 0.7 ± 0.02 a.u. ABTS radical scavenging capacity in percent was calculated according to following equation;

RSA % = [(A0-A1)/A0] x 100

Where A0 is the absorbance of ABTS solution includes methanol. A1 is the absorbance of ABTS solution in presence of extract or trolox as standard at various concentrations ranging from 0.019-0.617 mg/mL. After calculation of radical scavenging activity for trolox and plant extracts RSA % versus final concentrations were plotted and then trolox equivalent antioxidant capacity (TEAC) values were determined. TEAC values were calculated by dividing the slope of extract concentration versus RSA % graph to that of standard curve of trolox. All experiments were carried out with 6 different concentrations in quadruplicates and repeated as two different experiments.

2.2.3 Determination of Total Phenolic Content

Total phenolic content determination of the crude extract and their fractions were evaluated according to the Folin-Ciocalteu method proposed by Singleton and Rossi (1965) with some modifications. Instead of test cuvettes, 96 well-plates were used.

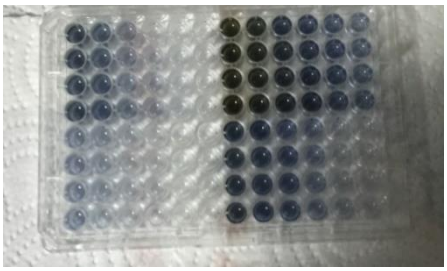


Figure 2.5: The color change in total phenolic content assay carried out in 96-well plates

Varying concentrations of gallic acid which was used as a standard, crude extracts and all fractions were dissolved in methanol. 10 µL of 1:4 Folin-Ciocaltaeu reagent with distilled water was put onto 10 µL of extracts with multi-channel pipette. After 10 min 200 µL of 2% Na_2CO_3 was added and 96-well plates were incubated for 30 minutes in dark, at room temperature. At the end of the reaction time, absorbance values at 750 nm were measured by Multiskan™ GO Microplate Spectrophotometer against blank, which constitutes only 10 µL of methanol. Final gallic acid concentrations prepared as 0.023, 0.0114, 0.006, 0.003, and 0.001 mg/mL versus absorbance graph was drawn as standard curve and it was used to calculate the amount of phenolic content of dry samples in terms of gallic acid equivalent (GAE). All experiments were carried out with 6 different concentrations in triplicates and repeated for two different experiments.

The Folin-Ciocalteu method is based on oxidation of phenolic groups by the Folin reagent (mixture of phosphomolybdic and phosphotungstic acids). The oxidation of Folin reagent produces a green-blue complex absorbed at 750 nm as seen in **Figure 2.5**. All experiments were carried out with 6 different concentrations in quadruplicates and repeated as two different experiments.

2.2.4 Determination of Total Flavonoid Content

Total flavonoid quantity in the extracts, were determined according to Zhishen et al. (1999) by the colorimetric reaction of sodium nitrite and aluminum chloride in the presence of flavonoids, with a modification to 96-well plates (**Figure 2.6**).

15 µL of various concentrations of crude extract and catechin standard, were mixed with 5 µL of 5% sodium nitrite (NaNO2). After incubating for 5 minutes at room temperature, 10 µL of 5% aluminium chloride (AlCl3) was added. 6 minutes later 35 µL of 1 M sodium hydroxide (NaOH) was added and mixtures were diluted with 135 µL of distilled water to complete the total volume up to 200 µL.

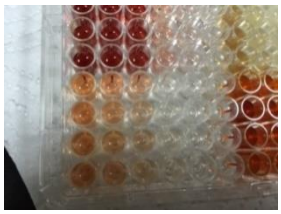


Figure 2.6: The color change in total flavonoid determination carried out in 96-well plates

The absorbance was measured against blank solution at 510 nm by using Multiskan™ GO Microplate Spectrophotometer (ThermoFisher Scientific, USA). Calibration curves of the standard and extracts were plotted as absorbance at 510 nm against concentration.

Total flavonoid content was calculated as catechin equivalent (CE) from catechin standard curve equation. All experiments were carried out with 6 different concentrations in quadruplicates and repeated as two different experiments.

Before cell culture studies, samples which are the most antioxidant and having the highest phenolic and flavonoid content, were decided. The rest of the experiments were performed with these extracts and/or fractions.

2.2.5 Cell Culture

2.2.5.1 Growth Conditions

3T3-L1, mouse fibroblast cells lines were grown in DMEM medium with 10 % calf bovine serum and antibiotic-antimycotic solution. They were incubated at 37 ºC in a 95 % humidified atmosphere of 5 % CO2. The cells were seeded at about 106 cells to 100 mm dishes, and never be allowed to become confluent.

2.2.5.2 Passaging

Sub-confluent 3T3-L1 cells were passaged when they reach 70 % confluence. If the cells were passaged at a later confluency stage, the cells would lose their

ability to differentiate into adipocytes. The medium was discarded using pipettes from the petri without disturbing attached cells. 2 ml of Trypsin/EDTA was added and incubated at 37oC for 2-5 minutes to allow the cells detached. Trypsin was inactivated by adding 1 mL of calf bovine serum. Then, detached cells were put in a 15 mL of sterile falcon and centrifuged at 1000 g for 5 min. Then the cells in the pellet were re-suspended and passaged to new petri dishes.

2.2.5.3 Freezing and Thawing

Freezing medium contains 5% DMSO was prepared with complete growth medium. For every 1 million of cells, 1 ml of freezing medium was used. After trypsinization of the cells, cell suspension was centrifuged and supernatant of the media was wasted. Remaining pellet was re-suspended with enough volume of freezing medium. Then it was put into the cryo-vials.

It was really important to store the cryo-vials at -20 °C for 2 hours, to freeze the cells gradually. Then they are placed in –86 °C freezer overnight. Next day the cryo-vials were moved to a liquid nitrogen containing tank which have -196 °C temperature, for long-term storage.

While thawing the cells, cryo-vials were placed into a water bath at the temperature of 37 °C. It is very important that cells should be exposed to freezing medium as little time as possible prior to freezing because of DMSO content. In 1-2 minutes, the medium was dissolved to transfer into T-25 tissue culture flasks. In the next day, media was changed to get rid of DMSO effect on cells.

2.2.5.4 Cell Viability

Cell membrane integrity and numbers of living and dead cells were evaluated by trypan blue dye exclusion method. Basic principle of this method is that trypan blue stains dead cells and cannot penetrate into living cells.

Before each experiment cells were counted by adding 15 μL of tryphan blue (0.5 % w/v) to 15 μL of cell suspension. 10 μL of this mixture was loaded in each of the two counting chambers of Juli-Br counting slides.

Juli-Br Cell Imaging System gives the numbers of viable and dead cells automatically. For the first experiments, to ensure the effectiveness of the machine; the cells were also counted by a haemocytometer under inverted microscope and the results were correlated, so Juli-Br system was used for counting before the differentiation of 3T3-L1 cells.

2.2.5.5 Differentiation of 3T3-L1 Cells

According to ATCC protocol chemically-induced differentiation of 3T3-L1 were done using single-component commercially-available reagents. Pre-adipocyte 3T3-L1 cells were grown in a desired culture vessel as described in Section 2.2.5.1. Then, they were seeded at a density of;

- 8×10^4 cells/well for 6 well plate
- 2×10^4 cells/well for 24 well plate
- 2×10^3 cells/well for 96 well plate

The cells were grown for 48 hours until the culture became 100% confluent. Cells were incubated as a confluent culture for another 48 hours. Growth medium was removed and an identical volume of differentiation medium was added. At this stage, the cells detached easily from the tissue culture vessel so gentle pipetting and handling was needed.

The assay was demonstrated according to ATCC protocol and the differentiation media was formulated as in the following: 90% Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1.0 μM Dexamethasone, 0.5 mM Methylisobutylxanthine (IBMX) and 1.0 μg/mL Insulin.

The cells were incubated in differentiation medium for 48 hours. After 48 hours, the differentiation medium was replaced with adipocyte maintenance medium which included:

1) 90% Dulbecco's Modified Eagle's Medium (DMEM)
2) 10% Fetal Bovine Serum
3) 1.0 μg/mL Insulin

The adipocyte maintenance medium was changed every 48 to 72 hours. The cells became fully differentiated between 7 to 15 days after induction, as representing lipid droplets (**Figure 2.7**).

In this study, all of the treatments were performed 10 days after induction with differentiation media.

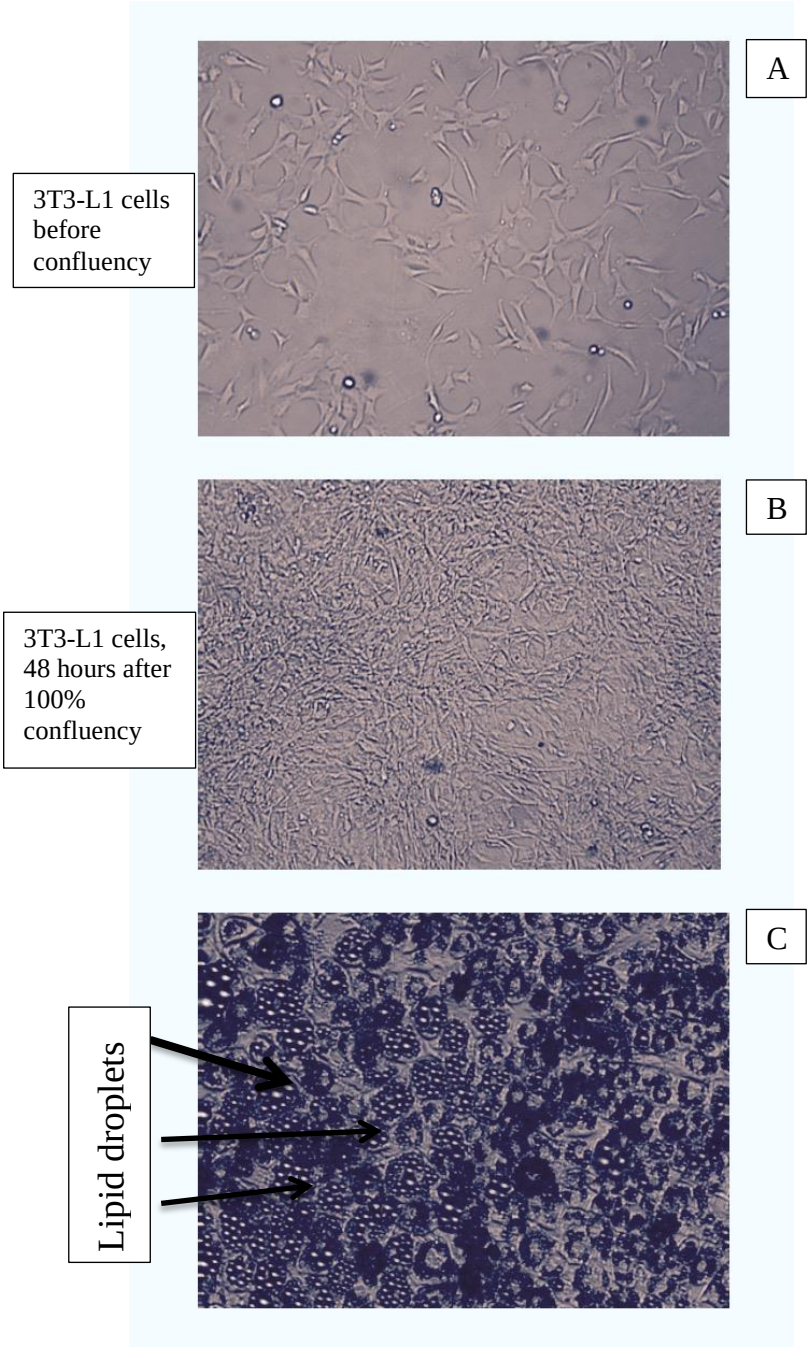


Figure 2.7: 1X images of 3T3-L1 cells taken by Juli-Br imaging system, before differentiation (A-B) and after differentiation (C)

*Lipid droplets indicating fully differentiation of 3T3-L1 cells at day 10.

2.2.6 Cytotoxicity

2.2.6.1 Preparation of the Samples

For cell culture studies all of the samples were dissolved in 10 % DMSO and 90 % DMEM as stock solutions. After optimization for 3T3-L1 cells DMSO is fixed to 0.1 % in every well and dishes. Stock solutions of crude extracts were prepared as 1, 10, 25, 50, and 100 mg/mL. Stock solutions of ethyl acetate fractions were prepared as 0.5, 1, 10, 25, 50 mg/mL.

2.2.6.2 XTT Method

The cytotoxic effects of the crude extracts and their fractions were investigated by using Cell Proliferation XTT Kit, to find the dose which was not lethal for differentiated 3T3-L1 cells. According to XTT method, metabolically active cells are able to reduce the tetrazolium salt of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) to orange colored compounds of formazan as displayed in **Figure 2.8**.

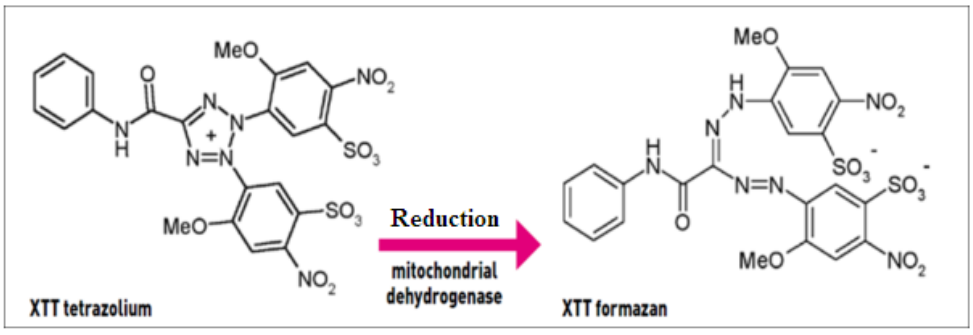


Figure 2.8: Reduction of XTT to form orange color formazan

The assay is based on the ability of metabolically active cells, indicating the higher activity of the mitochondrial enzymes, to reduce the tetrazolium salt XTT into orange colored compounds of formazan. The intensity of this dye is proportional to the number of metabolically active cells. According to the kit procedure, incubation for 2-24 hours was sufficient. During incubation time, an orange color was seen and its color intensity was read against a background at a wavelength of 450- 500 nanometer with an ELISA reader. In order to measure non-specific readings, wavelength of 630- 690 nanometer was subtracted from the 450-500 nanometer measurement.

100 µL of growth media was added to each well. The cells were incubated in a CO_2 incubator at 37°C. Each test included a complete medium blank without cells. Percent cell viability of the extract and its fraction was calculated with following equation:

$$\text{Cell viability (\%)} = \frac{\text{(av. OD of treated cells)- (av. OD of sample w/o cells)}}{\text{(av. OD of untreated cells)- (av. OD of control w/o cells)}} \times 100$$

Av.: Average
OD: Optical density

For XTT optimization; 3T3-L1 cells (about $2x10^3$ cells/well) were seeded into 96 well plates and differentiated. After differentiation 50 µL of XTT solution (0.1 mL activation solution mixed with 5 mL of XTT reagent) was added to the wells and at 0^{th}, 2^{nd}, 4^{th} hours absorbance was read and the appropriate time was determined as 2 hours.

After optimization, again 3T3-L1 cells were seeded and differentiated. 10 days after induction they were treated with 10-100-250-500 μg/mL, 1 mg/mL of crude extracts and 5-10-100-250-500 μg/mL of ethyl acetate fractions. Results of triple replicates were evaluated with standard deviations.

2.2.7 Fluorometric Cell-Based Assay for AMPK Phosphorylation

In order to determine the activity of AMPK, fluorometric cell-based assay was used. For this purpose, phosphorylation of AMPK was determined with EnzyFluo^{TM} AMPK phosphorylation assay kit (BioAssay Systems, USA). This kit is based on phosphorylation of AMPK on Thr-172 within the catalytic domain.

Phosphorylated AMPK in whole cells without cell lysate preparation measured by cell-based ELISA, and the signal was normalized to the amount of total protein. As displayed in **Figure 2.9**, the antibody recognizes α-subunit of the enzyme.

3T3-L1 cells grown and differentiated in clear black 96-well plates, were fixed. Using a fluorescent ELISA, AMPK phosphorylation was measured and then total protein was measured to calculate normalized phosphorylated AMPK (pAMPK) value in each well.

To avoid cross-contamination, pipette tips were changed between additions of each reagent or sample and separate reservoirs were used for each reagent. Samples were assayed in triplicate for optimization and treatment studies. For each plate a Protein Blank (no cells) in triplicate were included.

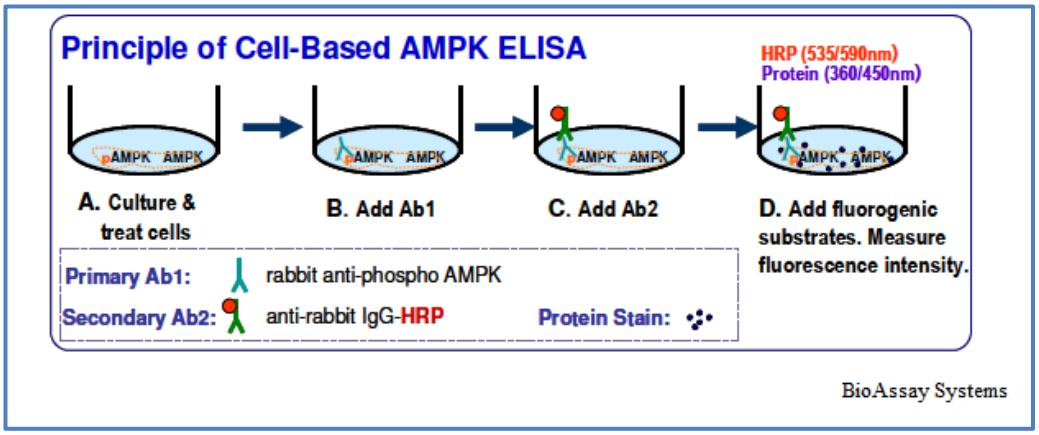


Figure 2.9: Principle of cell-based AMPK phosphorylation assay

For each tested sample a Sample Blank (cells with only Ab2) in triplicate was included to determine background fluorescence for total protein and phosphorylated AMPK, respectively. The plate was read at λex/em = 530/585 nm for phosphorylated AMPK (pAMPK) and at λex/em =360/450 nm for total protein.

$$\Delta\bar{F}_{pAMPK} = \bar{F}^{SAMPLE}_{pAMPK} - \bar{F}^{BLK}_{pAMPK}; \quad \Delta\bar{F}_{Prot} = \bar{F}^{SAMPLE}_{Prot} - \bar{F}^{BLK}_{Prot}$$

Normalized phosphorylated AMPK (pAMPK) is calculated as,

$$\text{Normalized pAMPK} = \frac{\Delta\bar{F}_{PAMPK} / \Delta\bar{F}_{Prot}}{(\Delta\bar{F}_{PAMPK} / \Delta\bar{F}_{Prot})_o}$$

Figure 2.10: Calculation of normalized phosphorylated AMPK (pAMPK) value where $(\Delta F_{pAMPK} / \Delta F_{Prot})_o$ is the control reference value (e.g. time zero in kinetic studies or untreated wells in drug potency studies.)

The mean fluorescence intensities were calculated with the formula given in **Figure 2.10**. The mean fluorescence of the sample blank wells was subtracted from the fluorescence value of the sample well to yield ΔF values for the phosphorylated AMPK (ΔF_{pAMPK}) at 530/585nm and for the total protein (ΔF_{Prot}) at 360/450nm.

2.2.8 Determination of mRNA Expression Level

2.2.8.1 Total RNA Isolation

Cell line was seeded onto 60 mm dishes for RNA isolation. After the cells were differentiated they were treated with the extracts and fractions at a concentration of increase the activity of AMPK enzyme.

After 48 hours treatment, total RNA was extracted with an RNA isolation kit, Life Science innuPREP RNA mini kit, AnalytikJena. At the end of treatment media removed by aspiration and the cells were washed twice with PBS. Cells were lysed with 350 μL of lysis solution. Lysates were fractionated with chloroform to get rid of the excess fat. 200 μL of chloroform were added to the tubes and were shaken vigorously. They were incubated for half an hour at room temperature and the upper aqueous phase containing RNA was taken. After fractionation this aqueous phase was transferred to D-spin column to remove DNA content of the cells and for 2 min they were centrifuged (12000 rpm). Equal amount of ethanol was added to the remaining eluent and mixed with equal amount of 70% of ethanol by gently pipetting and it was transferred into R-spin column and centrifuged for 2 min, 12000 rpm. After discarding remaining eluent, the column washed with HS and LS washing solution

respectively. The column was allowed to dry for 3 min, at12000 rpm. Finally, elution buffer was added to the column and centrifuged for 1 min, at 8000 rpm. Total RNA content and purity was determined with AlphaSpec Nano-drop (Alpha Innotech) at 260 nm. The ratio of OD260/OD280 between 1.8 and 2.2 indicated the purity of RNA.

2.2.8.2 cDNA Synthesis

cDNA was synthesized according to the eight version of Transcriptor High Fidelity cDNA synthesis kit (Roche, Germany). All the reagents were thawed and kept on ice.

Table 2.2: cDNA synthesis kit components (Roche)

Component	Volume	Final concentration
Template RNA	variable	1000 ng
Anchored-oligo $(dT)_{18}$ primer, 50 pmol/µL (vial 5)	1 µL	2.5 µM
Water (PCR-grade)	Variable	to make total volume=13 µL
Total volume	**13 µL**	
Transcriptor Reverse Transcriptase Reaction Buffer, 5x conc. (vial 2)	4 µL	1 x (8 mM $MgCl_2$)
Protector Rnase Inhibitor 40 U/ µL (vial 3)	0.5 µL	20 U
Deoxynucleotide Mix, 10 Mm each (vial 4)	2 µL	1 mM each
Transcriptor Reverse Transcriptase 20 U/ µL (vial 1)	0.5 µL	10 U
Final volume	**20 µL**	

For cDNA synthesis reaction mixture was prepared as seen in the **Table 2.2.** Mixture was incubated at 55 ^{o}C, for 30 min. Reverse transcriptase was inactivated for 5 min, at 85 ^{o}C in a conventional thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems). The resulting cDNA was kept at -20 ^{o}C.

2.2.8.3 Quantitative Real-Time Polymerase Chain Reaction

The expressions of AMPK subunits in 3T3-L1 cell line were analyzed by quantitative Real Time Polymerase Chain Reaction (qRT-PCR) using Roche LightCycler 480 (Germany). qRT-PCR experiments were done according to Light-Cycler® 480 SYBR Green I Master (Roche) kit, version 12. Prior to use 180 µL of RNAse/DNAse free water was added to the cDNA dilution (1:10). 100 µM stock primers were diluted as 1:100 with RNAse/DNAse free water.

Table 2.3: Reaction mixture in a well

Component	Volume
Water, PCR-grade	3 µL
Primer	2 µL
Master Mix, 2x concentration	10 µL
Total Volume	15 µL

As seen as in the **Table 2.3** reaction mixtures were prepared. 5 µL of cDNA template was added to each well. The multiwell-plate was sealed and run with Roche Lightcyler® 480. In order to detect any contamination, no template control (NTC) was used. As an internal standard, beta-actin gene was used.

The DNA amplification was carried out in a reaction mixture containing specific nucleotide sequence for related genes which are given in **Table 2.4**. Primer sequences were taken from Harvard Gene Bank except β-actin reference gene.

Table 2.4: Primers used in PCR experiments

Gene name	Forward Primer 5'→ 3'	Reverse Primer 5'→ 3'	bp
Prkaa1	CTCAGTTCCTGGAGAAAGATGG	CTGCCGGTTGAGTATCTTCAC	176
Prkab1	CTCACGAGAAGCCAGAATAACTT	TGTCCATCCACGAAGAACTTG	83
Prkag1	AATGAACACTTTCAAGAGACCCC	CCAACTTGGAACTTGTGGGAAT	103
β-actin	CACCAGGGTGTGATGGTGGGAAT	GGTCTTTACGGATGTCAACGTCACA	-

The qRT-PCR program profile was set to initial melting at 95 ^{o}C for 5 minutes, amplification repeated 45 times containing melting at 95 ^{o}C for 10 seconds, annealing at 60 ^{o}C for 10 seconds and extension at 72 ^{o}C for 10 seconds with a single fluorescent measurement. After cycling, melting curve program was set to 65-97 ^{o}C with a continious ramp rate of 0.11 ^{o}C/s. Relative quantification was done by using Ct (ΔΔCt) method;

1) ΔCt of target gene was normalized to the reference gene:
   $\Delta\mathbf{Ct} = Ct_{target} - Ct_{reference}$
2) ΔCt of the test sample (treated) was normalized to ΔCt of untreated one
   $\Delta\Delta\mathbf{Ct} = (Ct_{target} - Ct_{reference})_{treated} - (Ct_{target} - Ct_{reference})_{untreated}$
3) The fold difference in expression was calculated:
   $\mathbf{2^{-\Delta\Delta Ct}}$ = relative expression fold change

Melting curve analysis of the amplification product was done at the end of each amplification reaction to confirm the detection of a PCR product. Experiments were carried out as two independent experiments in duplicates.

2.2.9 Statistical Analysis

All values are expressed as mean ± standard error of the mean by Microsoft Excel. Statistical distributions and interpreting p values were calculated by using student's t-test. A statistically significant difference was considered to be at $p < 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Extraction of Plants

There have been several plants used for weight loss and appetite suppression in folk medicine of Anatolia. In this point of view; *C. limon*, *B. integerrima* and *T. bessarabicum* were selected for this purpose to investigate their effect as activators of AMPK of energy metabolism. Among the selected plants, lemon was separated as peel and juice, the other two started to be used dried.

Solvent extraction is the most commonly used procedure to handle the plant materials. It is quite efficient and easily applicable. Yield of solvent extraction depends on type of the solvents in different polarities, pH, duration of the extraction and temperature and also chemical composition of the sample being extracted (Pinelo, 2004). In this study, methanol was chosen as a solvent. Methanol is a widely used and effective organic solvent for the extraction of bioactive components. The use of methanol has been the highest yield of extract compared to other solvents used (Farhoosh, 2004). In a study, aqueous methanol (methanol:water, 70:30, v/v) solution was used to extract phenolic compounds because phenolics are extracted highly in polar solvents such as aqueous methanol as compared to absolute methanol (Siddhuraju, 2003).

In this study, the extraction of the selected plants was carried out as given in **Section 2.2.2.1**. The percent yields of methanol extraction of plant samples were given in **Table 3.1.**

Table 3.1: Percentage yields of the exracts

Plants	***C. limon* Peel**	***C. limon* Juice**	***B. integerrima***	***T. bessarabicum***
Total weight	47.6 g	800.0 g	80.0 g	120.0 g
Total extract	13.9 g	150.0 g	25.9 g	16.2 g
Yield	29.0 %	18.8 %	32.4 %	13.5 %

3.2 Fractionation of Crude Extracts

In this study, the solvent-solvent fractionation process was chosen for its simplicity and convenience (Obied, 2005) among different fractionation methods. Also, this approach is useful to understand the nature of bioactive compounds in plants/extracts and facilitate bioactivity guided fractionation.

Antioxidant compounds may be found connected to other compounds in plant materials such as glucose. And sometimes pigmentation of the plant can interfere with spectroscopic experimentations. Consequently, the pigmented lipoidal materials can be eliminated from the crude extract simply via fractionating with non-polar solvents such as hexane (Ramirez-Coronel, 2004), dichloromethane (Neergheen, 2006), or chloroform (Zhang, 2008).

The sequential fractionation of plant extracts were obtained by using solvents with different polarities (from non-polar to polar); which were hexane, chloroform, ethyl acetate, methanol-water (aqueous) respectively, and the resulting percentage yields were given in **Table 3.2.** Extraction/fractionation yields were recorded in the range of 1.9-40.2 %.

Table 3.2: Yields of fractions of the extracts

***C. limon* peel**	**Crude**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Aqueous**
Amount (g)	13.9	0.6	0.4	1.5	4.3
Yield (%)	-	4.3	2.9	10.8	30.9
***C. limon* juice**	**Crude**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Aqueous**
Amount (g)	20.4	0.1	0.1	4.9	8.2
Yield (%)	-	0.5	0.5	24.0	40.2
B. integerrima	**Crude**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Aqueous**
Amount (g)	20.9	0.7	0.4	11.1	4.6
Yield (%)	-	3.3	1.9	53.1	22.0
T. bessarabicum	**Crude**	**Hexane**	**Chloroform**	**Ethyl acetate**	**Aqueous**
Amount (g)	13.2	1.6	1.2	1.1	5.4
Yield (%)	-	12.1	9.1	8.3	40.9

3.3 Evaluation of Antioxidant Capacity

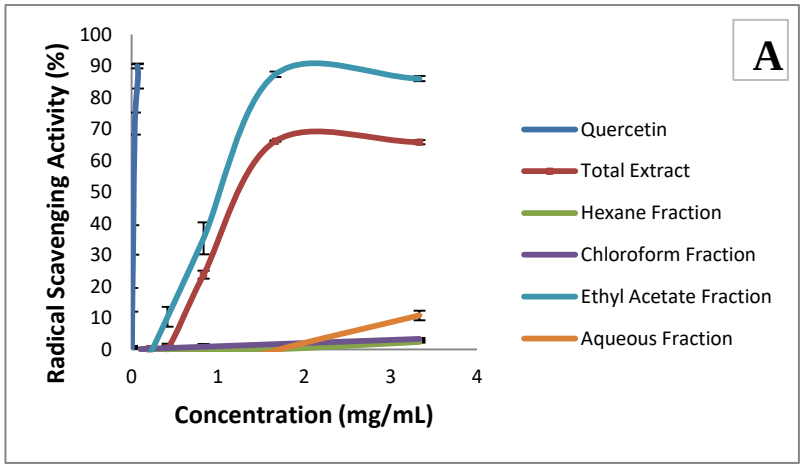
Antioxidant capacities were analyzed by two methods:

- DPPH radical scavenging capacity test
- ABTS radical scavenging capacity test

3.3.1 Free Radical Scavenging Capacity by DPPH

In this study, the free radical scavenging capacity of extracted samples and related fractions and their extracts were evaluated using DPPH method. DPPH (2,2-diphenyl-1-picrylhydrazyl) method is commonly used to determine antioxidant capacity for the plant extracts (Koleva, 2002). It is simple, quick, and economic besides it needs only a UV-vis spectrophotometer to perform the analysis. Radical scavenging activities (RSA) were shown in **Figure 3.1**. RSA (%) of the extracts and their fractions expressed as the 50 percent inhibitory concentration (IC_{50}) and lower the IC_{50} value means higher the antioxidant capacity. IC_{50} values were calculated according to RSA (%) values versus final concentration (mg/mL) plot of the crude extracts and fractionated extracts. IC_{50} values were shown in the **Table 3.3.**

As it was displayed in **Figure 3.1** total crude extracts and their ethyl acetate fraction exhibited the highest RSA (%). The peel of *C. limon* was found to be much more effective than its juice. RSA (%) values of *B. integerrima* and *T. bessarabicum* were even more effective than *C. limon* peel and juice, significantly.



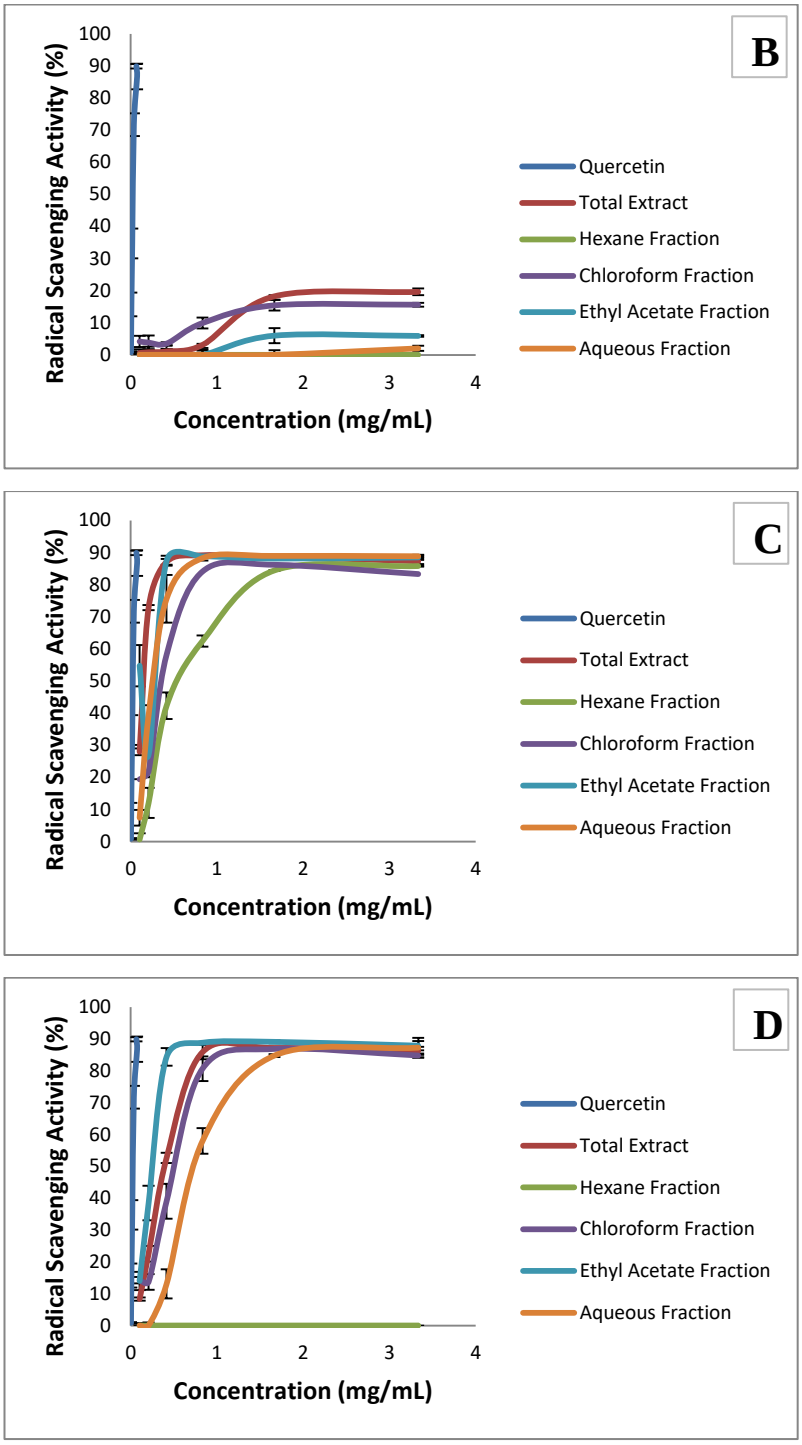


Figure 3.1: DPPH radical scavenging capacity (% RSA) of A) *C. limon* peel B) *C. limon* juice C) *B. integerrima* D) *T. bessarabicum*. Each point is the mean of quadruplicate data from two different sets of experiments, taken by 96-well microplate reader, absorbance at 517 nm.

A well-known phenolic standard, quercetin, widely used in DPPH experiments as a positive control, was chosen in this study because of its high antioxidant capacity. IC_{50} value of quercetin was determined as 0.002 ± 0.001 mg/mL.

As it was shown in **Table 3.3**; the IC_{50} values for *C. limon* peel has been found to be 0.19 ± 0.01, 0.92 ± 0.09, 0.29 ± 0.01, 0.15 ± 0.05, 0.72 ± 0.20 for crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction; respectively. Crude extract and ethyl acetate fraction of *C. limon* peel showed the highest antioxidant capacity with their lowest IC_{50} values.

The IC_{50} values for *C. limon* juice has been found to be 0.84 ± 0.15, 2.36 ± 0.38, 0.92 ± 0.17, 0.73 ± 0.08, 1.04 ± 0.16 for crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction; respectively. Again crude extract and ethyl acetate fraction of *C. limon* juice showed the highest antioxidant capacity with their lowest IC_{50} values same as with *C. limon* peel.

It was found that the IC_{50} values for *B. integerrima* to be 0.05 ± 0.02, 0.12 ± 0.01, 0.19 ± 0.08, 0.06 ± 0.01, 0.18 ± 0.09 for crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction; respectively. Crude extract and ethyl acetate fraction of *B. integerrima* showed the highest antioxidant capacity with their lowest IC_{50} values.

T. bessarabicum has been found to have IC_{50} values of 0.09 ± 0.02, 0.98 ± 0.07, 0.16 ± 0.06, 0.07 ± 0.02, 0.14 ± 0.03 for crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction; respectively. Crude extract and ethyl acetate fraction of *T. bessarabicum* showed the highest antioxidant capacity with their lowest IC_{50} values.

As a result, except for the hexane, all the fractionated extracts revealed a significant antioxidant effect against the DPPH radical. Among the samples, the lowest IC_{50} value, which means the highest radical scavenging capacity, was observed for crude extracts and their ethyl acetate fractions. It means that crude extracts and ethyl acetate fractionated extracts could be considered quite effective as DPPH radical scavengers.

Recently, in a report by Rekha et al. (2015), it was stated that among citrus fruits, *C. sinensis*, *C. limon* and *C. reticulate; C. limon* exhibited RSA % as 95 % capacity in 100 μL/mL concentration. In another study, aqueous fraction extract of *C. limon* juice was found to have IC_{50} value of 11.15 ± 3.08 mg/mL (Guimarães, 2010) which was very similar to the result of DPPH activity that was performed in this thesis study.

Serteser et al., (2009) studied some berberis species for their antioxidant properties and they found that water-methanol extracts of *Berberis crataegina* DC., *Berberis integerrima* Bunge, and *Berberis vulgaris* L. had IC_{50} values of 1.345, 1.412, 1.456 mg sample / mg DPPH; respectively.

In a report in 2015, Rezaeian et al. showed some Berberis species had IC_{50} DPPH inhibition values of 0.21±0.02 mg/mL for *Berberis integerrima* and 0.45±0.03 mg/mL for *Berberis vulgaris* (Rezaeian, 2015) that were very comparable results with this study although they had used different Berberis species.

It was not encountered with a study, previously reported about DPPH radical scavenging capacity of *T. bessarabicum*.

Table 3.3 Comparison of DPPH IC_{50} (mg/mL) results for *C. limon* peel, *C. limon* juice, *B. integerrima* and *T. bessarabicum* crude extracts and their fractions.

***C. limon* peel**	**DPPH IC_{50} (mg/mL) ± SD***
Crude extract	0.19 ± 0.01
Hexane fraction	0.92 ± 0.09
Chloroform fraction	0.29 ± 0.01
Ethyl acetate fraction	0.15 ± 0.05
Aqueous fraction	0.72 ± 0.20
***C. limon* juice**	**DPPH IC_{50} (mg/mL) ± SD***
Crude extract	0.84 ± 0.15
Hexane fraction	2.36 ± 0.38
Chloroform fraction	0.92 ± 0.17
Ethyl acetate fraction	0.73 ± 0.08
Aqueous fraction	1.04 ± 0.16
B.integerrima	**DPPH IC_{50} (mg/mL) ± SD***
Crude extract	0.05 ± 0.02
Hexane fraction	0.12 ± 0.01
Chloroform fraction	0.19 ± 0.08
Ethyl acetate fraction	0.06 ± 0.01
Aqueous fraction	0.18 ± 0.09
T.bessarabicum	**DPPH IC_{50} (mg/mL) ± SD***
Crude extract	0.09 ± 0.02
Hexane	0.98 ± 0.07
Chloroform	0.16 ± 0.06
Ethyl acetate	0.07 ± 0.02
Aqueous	0.14 ± 0.03
Quercetin	**0.002 ± 0.001**

DPPH IC_{50}: Inhibitory concentration of crude extracts and their fractions required for scavenging 50% of DPPH radical. *Mean of two independent experiments in quadruplicates. *Absorbance values obtained with 96-well microplate reader at 517 nm.

3.3.2 Free Radical Scavenging Activity by ABTS

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay is widely used for determination of antioxidant capacity of plant extracts. Among the assays of total antioxidant capacity determination in plant extracts, there are some advantages of ABTS method such as its accuracy and its easy application. ABTS method is very similar to DPPH radical scavenging capacity assay and it is more consistent to use more than one antioxidant methods for reliability.

Antioxidant capacities of the crude extracts and their fractions were determined according to their radical scavenging capacity with ABTS method as described previously in **Section 2.2.2.2.** In this method, trolox was used as a standard and the concentration curve for standardized trolox was given in **Figure 3.2**.

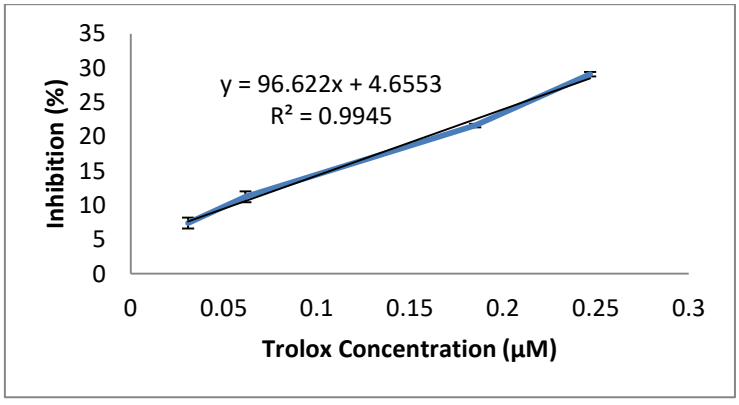


Figure 3.2: Calibration curve of radical scavenging capacity (inhibition) in percent versus concentration of trolox standard by using 96-well microplate reader, at 734 nm.

The linear equation driven from the concentration curve of trolox was used to calculate TEAC (trolox equivalent antioxidant concentration) values for the samples of crude extracts and their fractionated extracts. The inhibition curve slope of samples were divided to the slope of trolox standard in order to calculate the TEAC values of each sample in terms of μM Trolox equivalent/mg extract. Higher the TEAC value means higher the antioxidant capacity.

As listed in **Table 3.4**, TEAC values calculated for *C. limon* peel were found as 1.46 ± 0.10, 0.11 ± 0.01, 1.39 ± 0.06, 2.13 ± 0.02 and 0.99 ± 0.04 μM for crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction; respectively. Among the studied fractions, crude extract and ethyl acetate fraction of *C. limon* peel revealed the highest TEAC value indicating that they have the highest radical scavenging capacity, compatible with their DPPH results.

It was found that, TEAC values calculated for *C. limon* juice were found as 0.27 ± 0.01, 0.12 ± 0.01, 0.12 ± 0.01, 0.19 ± 0.03 and 0.09 ± 0.01 μM for crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction; respectively. Among the investigated fractions, crude extract and ethyl acetate fraction of *C. limon* juice revealed the highest TEAC value indicating that they have the highest radical scavenging capacity. All the TEAC values were compatible with DPPH results.

For crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction for *B. integerrima*; TEAC values were calculated and were found as 5.06 ± 0.07, 1.42 ± 0.07, 2.26 ± 0.04, 2.75 ± 0.06 and 2.11 ± 0.03 μM; respectively. Among those, crude extract and ethyl acetate fraction of *B. integerrima* again revealed the highest TEAC value indicating that they have the highest radical scavenging capacity. All the TEAC values were compatible with DPPH results.

T. bessarabicum shown to have TEAC values as 1.43 ± 0.03, 0.46 ± 0.02, 1.11 ± 0.03, 4.20 ± 0.10 and 0.71 ± 0.04 μM for crude extract, hexane fraction, chloroform fraction, ethyl acetate fraction, aqueous fraction; respectively. Among the investigated fractions, crude extract and ethyl acetate fraction of *T. bessarabicum* revealed the highest TEAC value indicating that they have the highest radical scavenging capacity. All the TEAC values were compatible with DPPH results.

In the study of Di Vaioa et al. (2010); ethanolic extracts of lemon peels were examined for their antioxidant capacity by the method of ABTS. The results indicated that different cultivars of lemons showed different antioxidant capacities. It was concluded that although they are good radical scavengers the results can differ in the diverse cultivars of the same species.

There are lots of reports about antioxidant activities of citrus species. According to a study of Barreca et al., 2011, ability of *C. limetta* juice as a natural antioxidant was evaluated by means of $ABTS^{\bullet+}$ radical scavenging. The results showed that, flavonoid-rich *Citrus limetta* revealed a significant scavenging capacity towards $ABTS^{\bullet+}$ radical cation with the valued of 1446 ± 30 μM trolox equivalents (TE).

There was no study regarding the antioxidant capacity of *B. integerrima*. However, there was a study about *B. vulgaris L.* species done by Yildiz et al. (2014) with 19 genotypes grown in the Çoruh valley, of northeastern Anatolia. In this study, the total antioxidant capacity of barberry fruit extracts was determined with the methods of FRAP and TEAC.

No study was reported previously about ABTS radical scavenging capacity of *T. bessarabicum* extract.

Table 3.4: Trolox equivalent antioxidant capacities (TEAC) of the extracts

***C. limon* peel**	**TEAC value (µM Trolox ± SD*)**
Crude extract	1.46 ± 0.10
Hexane fraction	0.11 ± 0.01
Chloroform fraction	1.39 ± 0.06
Ethyl acetate fraction	2.13 ± 0.02
Aqueous fraction	0.99 ± 0.04
***C. limon* juice**	**TEAC value (µM Trolox ± SD*)**
Crude extract	0.27 ± 0.01
Hexane fraction	0.12 ± 0.01
Chloroform fraction	0.12 ± 0.01
Ethyl acetate fraction	0.19 ± 0.03
Aqueous fraction	0.09 ± 0.01
B. integerrima	**TEAC value (µM Trolox ± SD*)**
Crude extract	5.06 ± 0.07
Hexane fraction	1.42 ± 0.07
Chloroform fraction	2.26 ± 0.04
Ethyl acetate fraction	2.75 ± 0.06
Aqueous fraction	2.11 ± 0.03
T. bessarabicum	**TEAC value (µM Trolox ± SD*)**
Crude extract	1.43 ± 0.03
Hexane fraction	0.46 ± 0.02
Chloroform fraction	1.11 ± 0.03
Ethyl acetate fraction	4.20 ± 0.10
Aqueous fraction	0.71 ± 0.04

TEAC value: Radical scavenging capacity µM equivalents of trolox/mg of crude extract and fractions.

* Mean of two independent experiments in quadruplicates.

**Absorbance was measured at 734 nm with 96-well microplate reader.

3.4 Determination of Total Phenolic Content

Total phenolic contents of selected plant extracts and their fractions were determined by the method of Singleton and Rossi (1965) with some modifications, the Folin-Ciocalteu reagent assay. The calibration curve of gallic acid against absorbance value at 750 nm observed by 96-well microplate reader was plotted as shown in **Figure 3.3.** The equation extracted from this graph was used to calculate the phenolic content of the extracts in terms of gallic acid equivalent value (GAE).

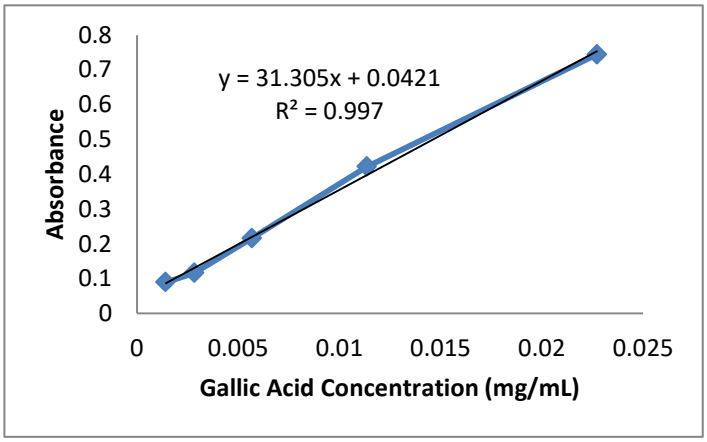


Figure 3.3: Calibration curve for gallic acid standard. Experiments were performed as two independent experiments in quadruplicates. Measurements were performed at 750 nm with a 96-well microplate reader.

The calibration curve equation was found as y = 31.305x + 0.0421 with R^2 = 0.9970. The results were given in mg phenolics in extract as equivalents of gallic acid (GAE) in mg (**Table 3.5).** The crude extract and ethyl acetate fraction of *C. limon* peel, with a value of 23.9 ± 1.8 and 35.41 ± 5.40 GAE (µg

equivalents of gallic acid/mg of plant extract) respectively, was shown to have high total phenolic content among the other fractions of the lemon peel.

It was shown that the crude extract and ethyl acetate fraction of *C. limon* juice, with a value of 7.21 ± 0.51 and 4.92 ± 0.35 GAE (µg equivalents of gallic acid/mg of plant extract) respectively, had high total phenolic content among the other fractions of the lemon juice.

The crude extract and ethyl acetate fraction of *B. integerrima*, with a value of 68.30 ± 8.41 and 73.72 ± 10.77 GAE (µg equivalents of gallic acid/mg of plant extract) respectively, was shown to have high total phenolic content among the other fractions of the *B. integerrima.*

With a value of 36.82 ± 4.84 and 61.03 ± 9.67 GAE (µg equivalents of gallic acid/mg of plant extract) respectively, the crude extract and ethyl acetate fraction of *T. bessarabicum* displayed high total phenolic content among the other fractions of the *T. bessarabicum.*

Ghasemi et al. (2009) reported that methanolic extract of *C. limon* peel had 131.0 mg gallic acid equivalent/g of extract powder.

Another research group (Fejzić, 2014) stated that Citrus fruits from Rutaceae family, are important source of phenolic compounds, responsible for antioxidant and for some other biological activities. Five different types of citrus juice extracts and peels which were lemon, pink grapefruit, tangerine, white grapefruit and orange; were analyzed for their antioxidant activities and total phenolic contents. Total phenolic content was determined by Folin-Ciocalteu method, and the values varied from 0.192 ± 0.015 mg GAE/mL for the peel of white grapefruit to 0747 ± 0098 mg GAE/mL for the juice of white grapefruit.

Table 3.5: Total phenolic contents of extracts as expressed in gallic acid equivalents (GAE)

***Citrus limon* peel**	**Total Phenol GAE (μg/mg) ± SD***
Crude extract	23.9 ± 1.8
Hexane fraction	7.01 ± 0.82
Chloroform fraction	13.60 ± 4.90
Ethyl acetate fraction	35.41 ± 5.40
Aqueous fraction	12.42 ± 4.33
***C. limon* juice**	**Total Phenol GAE (μg/mg) ± SD***
Crude extract	7.21 ± 0.51
Hexane fraction	1.73 ± 0.52
Chloroform fraction	3.91 ± 0.92
Ethyl acetate fraction	4.92 ± 0.35
Aqueous fraction	3.73 ± 0.26
B. integerrima	**Total Phenol GAE (μg/mg) ± SD***
Crude extract	68.30 ± 8.41
Hexane fraction	7.62 ± 1.65
Chloroform fraction	34.03 ± 8.51
Ethyl acetate fraction	73.72 ± 10.77
Aqueous fraction	54.50 ± 5.04
T. bessarabicum	**Total Phenol GAE (μg/mg) ± SD***
Crude extract	36.82 ± 4.84
Hexane fraction	2.02 ± 0.25
Chloroform fraction	24.68 ± 4.52
Ethyl acetate fraction	61.03 ± 9.67
Aqueous fraction	22.12 ± 6.84

TP GAE: Total phenolic contents μg equivalents of gallic acid/mg of plant extract.

*Measurements were performed by microplate reader, at 750 nm.

*Mean of two independent experiments in quadruplicates

There was no study indicating phenolic content of *B. integerrima* and *T. bessarabicum* species. But there were some studies about other species of these families. According to a study of Bonesi et al. (2013) total phenolic content of *B. aetnensis* and *B. libanotica* were found to have 27.4 ± 0.9 and 69.0 ± 0.8 milligrams of chlorogenic acid equivalents per gram of plant materials, respectively, similar to the results of *B. integerrima* which was used in this study.

3.5 Determination of Total Flavonoid Content

Total flavonoid content was determined by the reaction between sodium nitrite and aluminum chloride, which results in a colored flavonoid-aluminum complex and monitored at 510 nm using the 96-well microplate reader spectroscopically.

Total flavonoid content was expressed as catechin equivalents (CE) in milligram per crude extract and their fractions in milligram. The calibration curve of catechin standard was displayed in **Figure 3.4**. Equation of this curve was $y = 7.9937x + 0.0473$ with $R^2 = 0.9894$.

Total flavonoid content of crude extracts and their fractions were given in **Table 3.6**. Crude extracts and ethyl acetate fractions of selected plants were found to have high total flavonoid content.

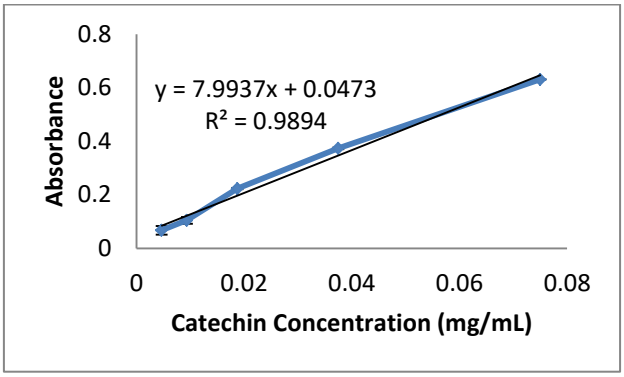


Figure 3.4: Calibration curve of catechin standard (Experiments were performed as two independent experiments in quadriplicates and measurements were performed by 96-well microplate reader at 510 nm).

The crude extract and ethyl acetate fraction of *C. limon* peel, with a value of 19.3 ± 2.6 and 28.2 ± 4.4 CE (µg equivalents of catechin/mg of plant extract) respectively, were found to have high total flavonoid contents compared to the other fractions of the same plant.

It was found that; crude extract and ethyl acetate fraction of *C. limon* juice, with a value of 3.1 ± 0.4 and 4.1 ± 0.3 CE (µg equivalents of catechin/mg of plant extract) respectively, had higher total flavonoid content than the other fractions of the same plant extract.

Again, with a value of 185.8 ± 13.5 and 216.9 ± 12.2 CE (µg equivalents of catechin/mg of plant extract), crude extract and ethyl acetate fraction of *B. integerrima* respectively were found to have high total flavonoid content among the other fractions of the same plant extract.

Table 3.6: Total flavonoid content of extracts

***C. limon* peel**	**Total flavonoid CE (µg/mg) ± SD***
Crude extract	19.3 ± 2.6
Hexane fraction	10.2 ± 3.8
Chloroform fraction	9.8 ± 0.7
Ethyl acetate fraction	28.2 ± 4.4
Aqueous fraction	11.4 ± 2.5
***C. limon* juice**	**Total flavonoid CE (µg/mg) ± SD***
Crude extract	3.1 ± 0.4
Hexane fraction	0.9 ± 0.2
Chloroform fraction	1.2 ± 0.5
Ethyl acetate fraction	4.1 ± 0.3
Aqueous fraction	0.4 ± 0.1
B. integerrima	**Total flavonoid CE (µg/mg) ± SD***
Crude extract	185.8 ± 13.5
Hexane fraction	4.3 ± 1.4
Chloroform fraction	59.5 ± 8.9
Ethyl acetate fraction	216.9 ± 12.2
Aqueous fraction	11.7 ± 2.4
T. bessarabicum	**Total flavonoid CE (µg/mg) ± SD***
Crude extract	39.3 ± 3.9
Hexane fraction	3.6 ± 1.2
Chloroform fraction	26.5 ± 3.6
Ethyl acetate fraction	102.9 ± 8.1
Aqueous fraction	14.5 ± 2.5

CE: Total flavonoid contents µg equivalents of catechin/mg of plant extract.

Measurements were performed by 96-well microplate reader at 510 nm.

*Mean of two independent experiments in quadruplicates.

The crude extract and ethyl acetate fraction of *T. bessarabicum* revealed to have high total flavonoid content with a value of 39.3 ± 3.9 and 102.9 ± 8.1 CE (µg equivalents of catechin/mg of plant extract) respectively, among the other fractions of the same plant extract.

According to a study of Bonesi et al. (2013); total flavonoid content of *B. aetnensis* and *B. libanotica* were found to be 16.2 ± 0.6 and 31.5 ± 0.5 milligrams of quercetin equivalents per gram of plant materials, respectively.

Total flavonoid content of some citrus peels were found to be 49.2 ± 1.33 mg/g for *Citrus reticulata* Blanco, 39.6 ± 0.92 mg/g for *C. tankan* Hayata, 39.8 ± 1.02 mg/g for *C. reticulate x C. sinensis,* 46.7 ± 1.51 mg/g for *C. grandis* Osbeck, 48.7 ± 1.53 mg/g for *C. grandis* Osbeck CV, 41.0 ± 1.37 mg/g for *C. microcarpa,* 35.5 ± 1.04 mg/g for *C. sinensis* (L.) Osbeck, 32.7 ± 1.06 mg for *C. limon* (L.) Bur (Wang et al., 2008).

In general, crude extracts and ethyl acetate fractions with high radical scavenging capacity as well as high phenolic and flavonoid content were selected for the rest of the experiments to study their biological effects on AMPK as an activator.

3.6 Cell Culture

3.6.1 Growth Conditions and Viability of 3T3-L1 cells

The mouse embryonic fibroblast cell line 3T3-L1 is a model for lipid metabolism research because of the ability of the cells to differentiate into adipocytes with a chemical induction. Growth conditions were mentioned in **Section 2.2.5.1**.

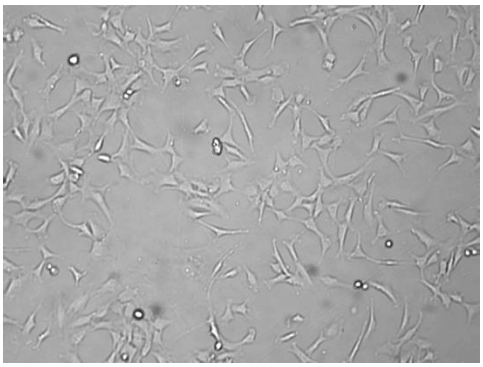


Figure 3.5: 1x image of sub-confluent 3T3-L1 cells taken by Juli-Br cell imaging system

3T3-L1 cells can lose their contact inhibition if they are densely cultured, therefore they were never let to reach 100% confluency throughout the study. They were passaged in every two days after they reached to 70% confluency as seen in **Figure 3.5**.

3.6.2 Differentiation of 3T3-L1 cells

3T3-L1 cells were differentiated into adipocytes. Prior to differentiation of 3T3-L1, cells were grown until they reach 100 % confluency. After confluency, they were fed for more 48 hours.

They were very fragile after induction with MDI chemicals (isobutylmethylxanthine, dexamethasone and insulin) for differentiation. In this stage, refreshing of the culture media should be carried out very gently.

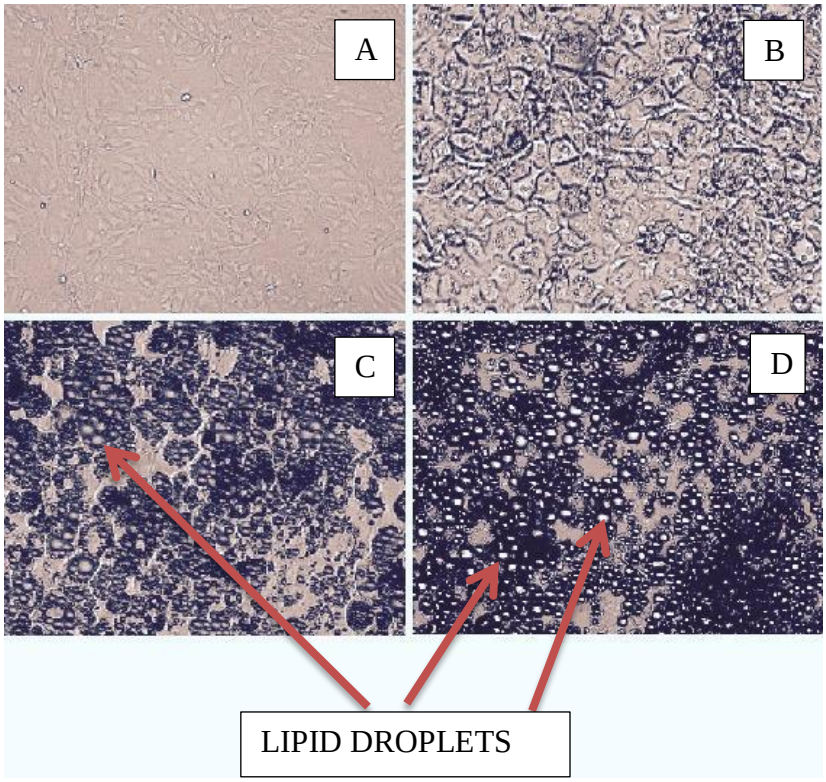


Figure 3.6: Images of 3T3-L1 cells in Juli-Br cell imaging system (1x) showing A) Before differentiation (Day 0) B) Second day after induction with differentiation media (Day 2) C) Day 8 D) Day 10 (*In the images of C and D, lipid droplets were indicating differentiation and all of the experiments were performed at day 10 when 3T3-L1 cells were fully differentiated.)

After 10 days of MDI induction, cells had observable lipid droplets indicating differentiation is fully completed. Full differentiation of 3T3-L1 cells was confirmed by observing large lipid droplets under the light microscope as displayed in **Figure 3.6** and finally cell treatments with the samples were performed.

Since differentiation media of 3T3-L1 cells contain insulin, metabolic activity of the cells increases. Consequently, excess fatty acids resulting from the high metabolic activity, secreted into the culture media and result in decrease of the pH consequently, cell death after 72 hours. Because of this reason all of the experiments were performed after 48 hours of incubation with the extracts.

3.6.3 XTT Assay

Cell viability of differentiated 3T3-L1 cell line, was determined with a rapid colorimetric assay using XTT as described in **Section 2.2.6.2**. Firstly, the time of XTT assay was optimized. It was found that two hours of XTT treatment was enough to measure absorbance at 475 nm.

Crude extracts and ethyl acetate fractions were dissolved in 10 % DMSO. DMSO used as a vehicle to increase the permeability of cells. Thereby, the entrance of the extracts into the cells was facilitated. Final DMSO concentration in each well was kept constant, as 0.1 %. Cell viability was measured at the end of 48 h of treatment with the extracts at the concentrations ranging between 5µg/mL - 1mg/mL on differentiated 3T3-L1 cell lines.

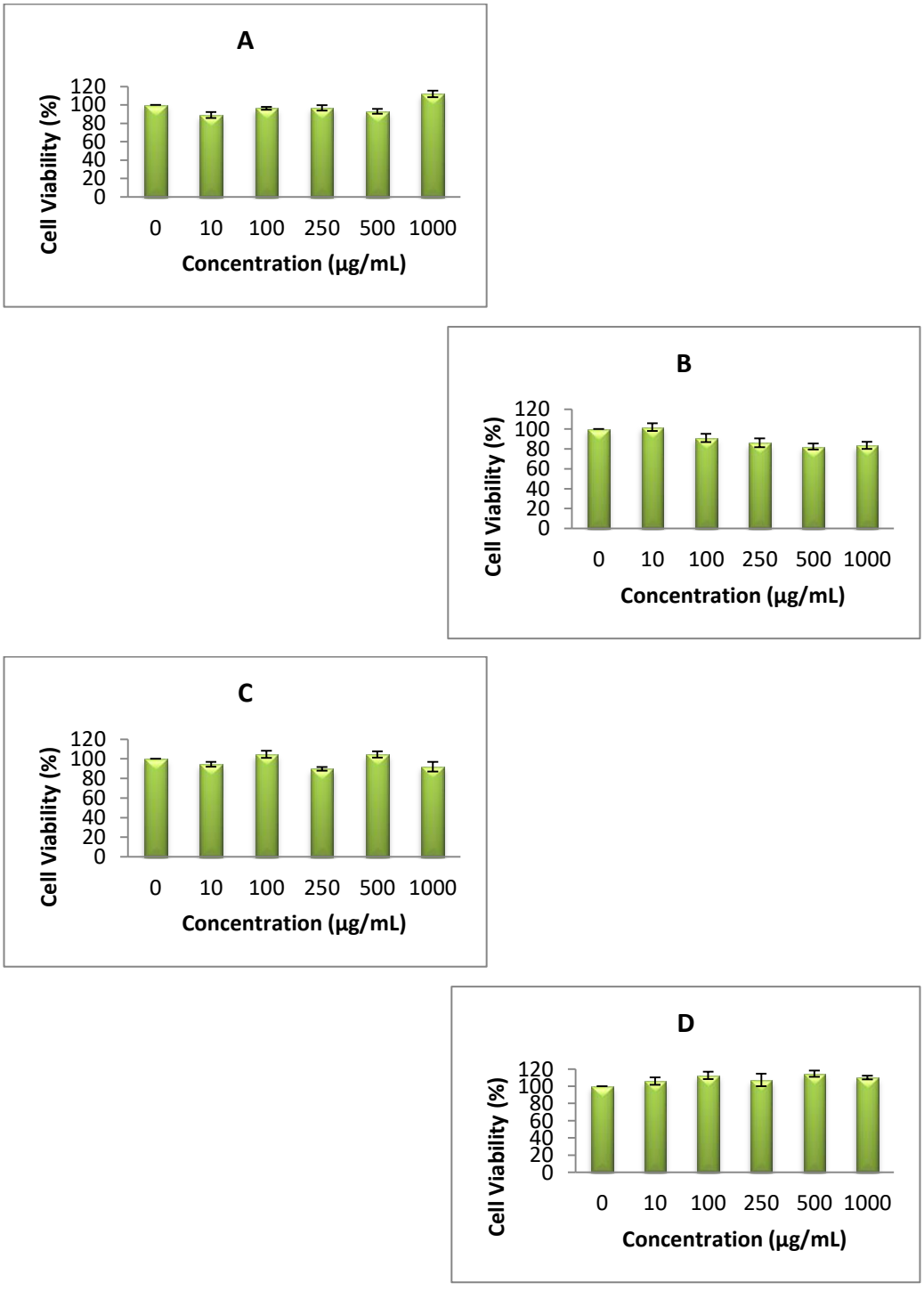


Figure 3.7: Cell viability of differentiated 3T3-L1 line, after 48 hours of treatment with A) *C. limon* peel B) *C. limon* juice C) *B. integerrima* D) *T. bessarabicum* crude extracts in different concentrations. *Measurements were completed with 96-well plates ELISA reader at 475 nm. *Mean value of two independent experiments in triplicates.

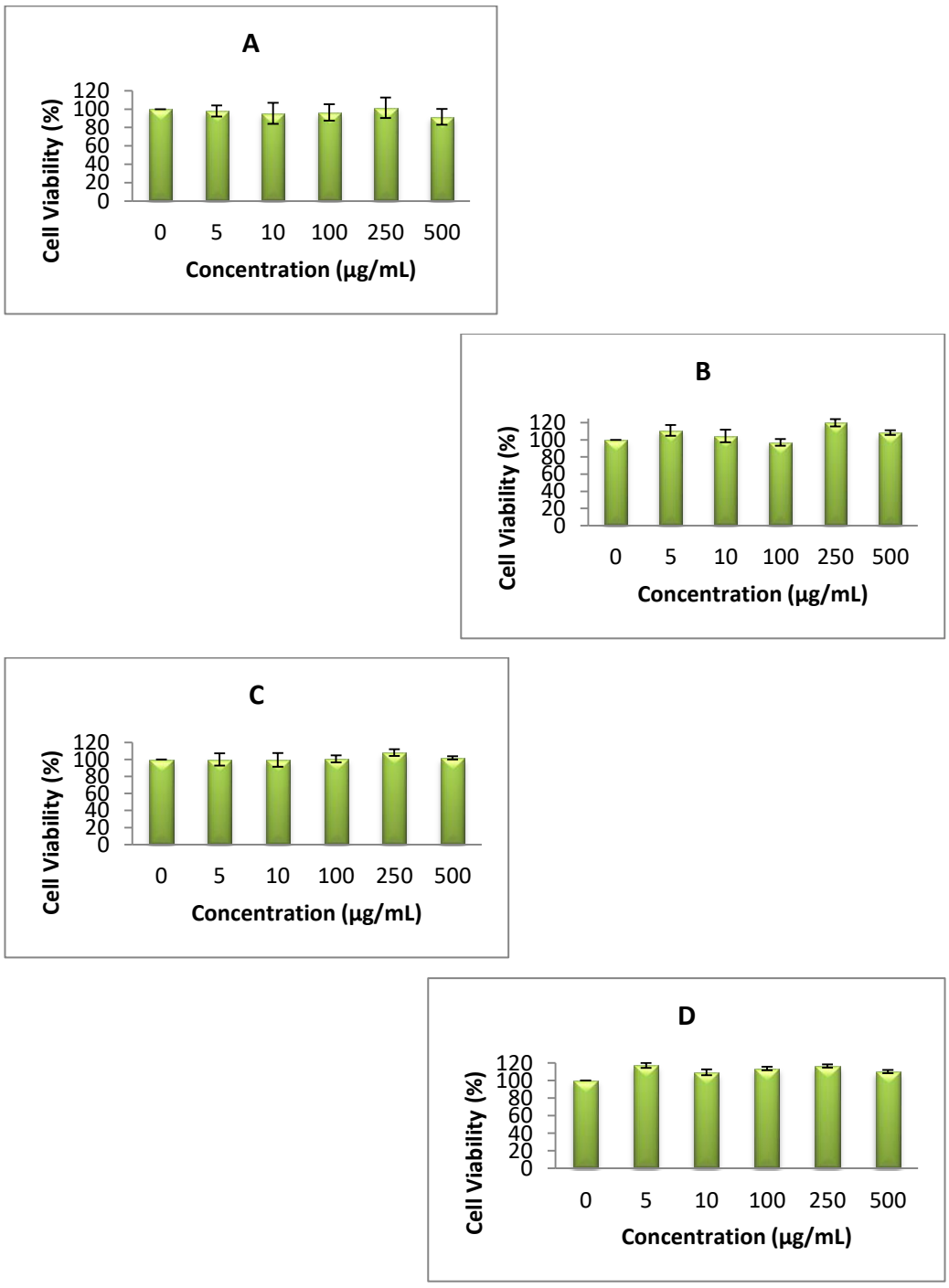


Figure 3.8: Cell viability of differentiated 3T3-L1 line, after 48 hours of treatment with A) *C. limon* peel B) *C. limon* juice C) *B. integerrima* D) *T. bessarabicum* ethyl acetate fractions in different concentrations. *Measurements were completed with 96-well plates ELISA reader at 475 nm. *Mean value of two independent experiments in triplicates.

Crude extracts, as illustrated in **Figure 3.7**, between the concentrations of 10 μg/mL and 1 mg/mL, did not show any cytotoxic effect on cell viability. Therefore, it was decided that treatments would be performed in these concentration range in enzyme activity and PCR assays.

For ethyl acetate fractions, as displayed in **Figure 3.8**, between 5-500 μg/mL, there was also no change in cell viability that means these fractions, in given concentration range, were not cytotoxic so they can be used in cell culture experiments.

Natural compounds such as resveratrol, berberine, quercetin, curcumin, and ginsenoside significantly were shown to speed up the phosphorylation of AMPK and these compounds are very important in the prevention or treatment of some diseases (Collins, 2007; Hwang, 2007; Fang, 2008; Cheng, 2006). In this thesis study, polyphenolic compounds found in natural sources were also searched for their effect on activation of AMPK.

3.6.4 Optimization of Fluorometric Cell-Based Assay for AMPK Phosphorylation

Cellular stress depletes ATP and cause to increase the concentration of AMP. AMP activates AMPK by allosteric activation, phosphorylation of alpha-subunit on Thr172, or inhibition of de-phosphorylation by phosphatases. Once AMPK activated it switches off anabolic pathway reactions such as gluconeogenesis; glycogen, fatty acid, triglyceride, cholesterol and protein synthesis and it switches on catabolic pathway reactions such as glycolysis, glucose uptake and fatty acid oxidation (Kola, 2008). For the very same reason, a fluorometric cell-

based assay was designed to follow this AMPK activation in this study. At the end, normalized phosphorylated AMPK (pAMPK) values were calculated.

In order to find the most effective dose of the extracts on AMPK as an activator, assay conditions were optimized. Plant extract concentrations which had been found to be effective in the previous cell culture studies in literature were considered and in the concentration of 1000 µg/mL crude extracts and 500 µg/mL ethyl acetate fractions were prepared in this study.

Table 3.7: Normalized phosphorylated AMPK (pAMPK) (%) of values of crude extracts in varying concentrations, in differentiated 3T3-L1 cells.

CONCENTRATION of *C. limon* peel	**Normalized pAMPK (%) ± SD***
10 µg	120.1 ± 6.1
100 µg	210.5 ± 4.7
250 µg	110.9 ± 7.6
500 µg	83.3 ± 3.2
CONCENTRATION of *C. limon* juice	**Normalized pAMPK (%) ± SD***
10 µg	85.7 ± 2.4
100 µg	95.2 ± 4.0
250 µg	90.8 ± 3.6
500 µg	97.1 ± 3.2
CONCENTRATION of *B. integerrima*	**Normalized pAMPK (%) ± SD***
10 µg	107.1 ± 4.3
100 µg	114.7 ± 4.3
250 µg	123.4 ± 4.0
500 µg	116.30 ± 3.5
CONCENTRATION of *T. bessarabicum*	**Normalized pAMPK (%) ± SD***
10 µg	107.8 ± 5.5
100 µg	102.7 ± 4.4
250 µg	94.4 ± 3.3
500 µg	95.6 ± 5.4
Untreated	**100**
Metformin (100 µM)	**240.8**

*Mean value of two independent experiments in triplicates and measurements were performed by fluorescent ELISA, at 530/585nm.

In cytotoxicity assays crude extracts concentrations of lower than 1000 µg/mL and ethyl acetate fraction concentrations of 500 µg/mL and lower were not cytotoxic on the differentiated 3T3-L1 cells. The extracts on differentiated 3T3-L1 cells were treated for 48 hours and the results were compared with the untreated negative control and with metformin which is an AMPK activator as positive control.

According to our results, as seen from the **Table 3.7**, among the extracts only 100 µg of *C. limon* peel crude extract was significantly effective and increased AMPK phosphorylation two times higher (210.49 ± 4.67 in percent) compared to untreated control, therefore the others were eliminated for the rest of the studies. Continued with the optimization of concentrations, for the *C. limon* peel crude extracts, which were prepared as 10, 25, 50 µg/mL for another trial of fluorometric AMPK phosphorylation. All of four ethyl acetate fractions were found to be effective on AMPK phosphorylation (Table 3.8). Dose dependence optimizations continued with the preparation of in between concentrations 10-50 µg/mL of ethyl acetate extract of samples for the AMPK phosphorylation assay.

Metformin, a synthetic derivative of guanide which is a natural product in Galega officinalis plant, was commonly used for the therapy of type 2 diabetes mellitus. Activation of AMPK by metformin suppresses the expression of a lipogenic transcription factor, SREBP-1. In a study (Zhou, 2001), metformin was shown to activate the AMP-activated protein kinase (AMPK) in vitro and in vivo. In a study of Hawley et al. (2002), the mechanism for this activation was studied. It was determined that metformin stimulated phosphorylation of Thr-172, on the catalytic α-subunit of AMPK in intact cells. In our study, metformin was also used as positive control as AMPK activator.

Table 3.8: Normalized phosphorylated AMPK (pAMPK) (%) values of ethyl acetate extracts in varying concentrations, in differentiated 3T3-L1 cells.

CONCENTRATION of *C. limon* peel	**Normalized pAMPK (%) ± SD***
5 μg	91.0 ± 4.3
10 μg	287.5 ± 8.2
100 μg	89.6 ± 5.6
250 μg	88.4 ± 7.4
CONCENTRATION of *C. limon* juice	**Normalized pAMPK (%) ± SD***
5 μg	90.9 ± 4.2
10 μg	90.3 ± 8.8
100 μg	222.3 ± 11.8
250 μg	108.5 ± 6.4
CONCENTRATION of *B. integerrima*	**Normalized pAMPK (%) ± SD***
5 μg	82.3 ± 4.5
10 μg	85.8 ± 2.4
100 μg	180.6 ± 7.1
250 μg	130.2 ± 9.2
CONCENTRATION of *T. bessarabicum*	**Normalized pAMPK (%) ± SD***
5 μg	81.3 ± 4.8
10 μg	410.5 ± 12.5
100 μg	93.9 ± 5.3
250 μg	81.7 ± 7.0
Untreated	**100**
Metformin (100 μM)	**240.8**

* Mean value of two independent experiments in triplicates and measurements were performed by fluorescent ELISA, at 530/585nm.

Currently, there is an increasing focus on the activation of AMPK using natural compounds in food supplements. Since AMPK is an important energy metabolism enzyme its phosphorylation by our selected plant extracts will be important for the design of new dietary supplements using plant originated natural compounds.

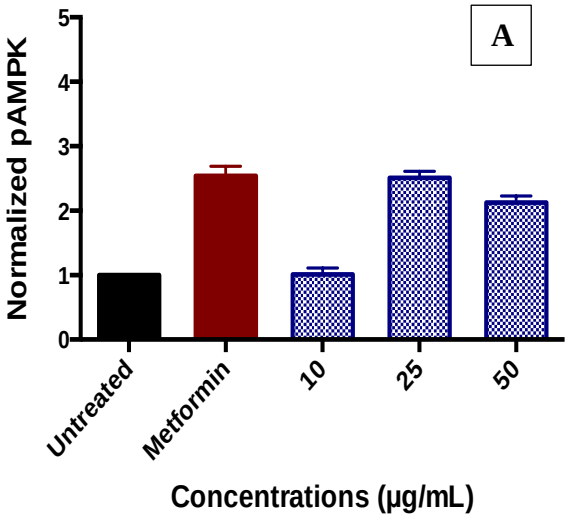
3.6.5 Fluorometric Cell-Based Assay for AMPK Phosphorylation

The activation effect of the extracts on AMPK phosphorylation (pAMPK) were re-evaluated after optimization studies. Normalized pAMPK values were calculated as described in **Section 2.2.7** and **Figure 2.9**. The phosphorylation of AMPK were found to be significant in differentiated 3T3-L1 cells treated by the plant extracts, as displayed in **Figure 3.9.**

According to AMPK phosphorylation assay, 25 µg/mL of *C. limon* peel crude extract, 10 µg/mL of *C. limon* peel ethyl acetate fraction, 25 µg/mL of *C. limon* juice ethyl acetate, 25 µg/mL of *B. integerrima* ethyl acetate fraction, 10 µg/mL of *T. bessarabicum* ethyl acetate fraction were found to be effective on enzyme phosphorylation.

A 10 µg/mL of *T. bessarabicum* ethyl acetate fraction revealed very high effect on AMPK activity with a normalized pAMPK value of 4.27 ± 0.01; which means that it increased enzyme phosphorylation 4 times higher than that of untreated control **(Figure 3.9).**

These extracts that were found effective on enzyme phosphorylation, were used for further PCR experiments to see the enzyme phosphorylation is coming whether from post-translational modification or from transcription of AMPK genes.


A
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Untreated
Metformin
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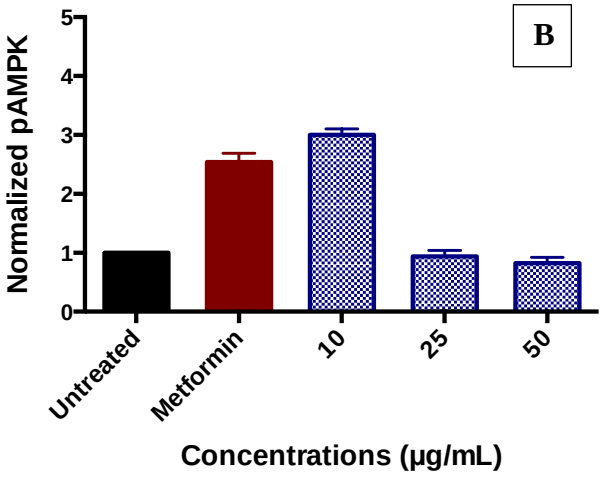
50
Concentrations (µg/mL)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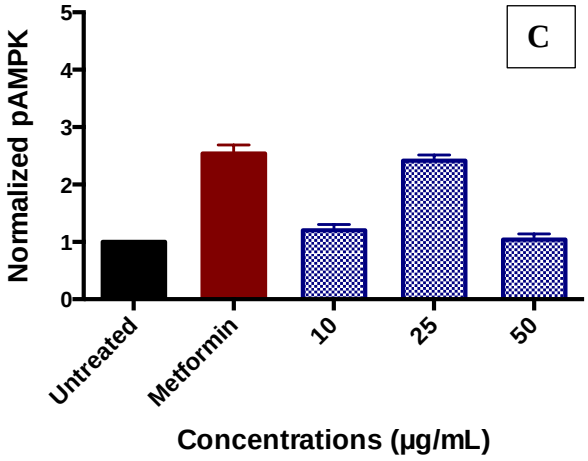
Concentrations (µg/mL)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Concentrations (µg/mL)

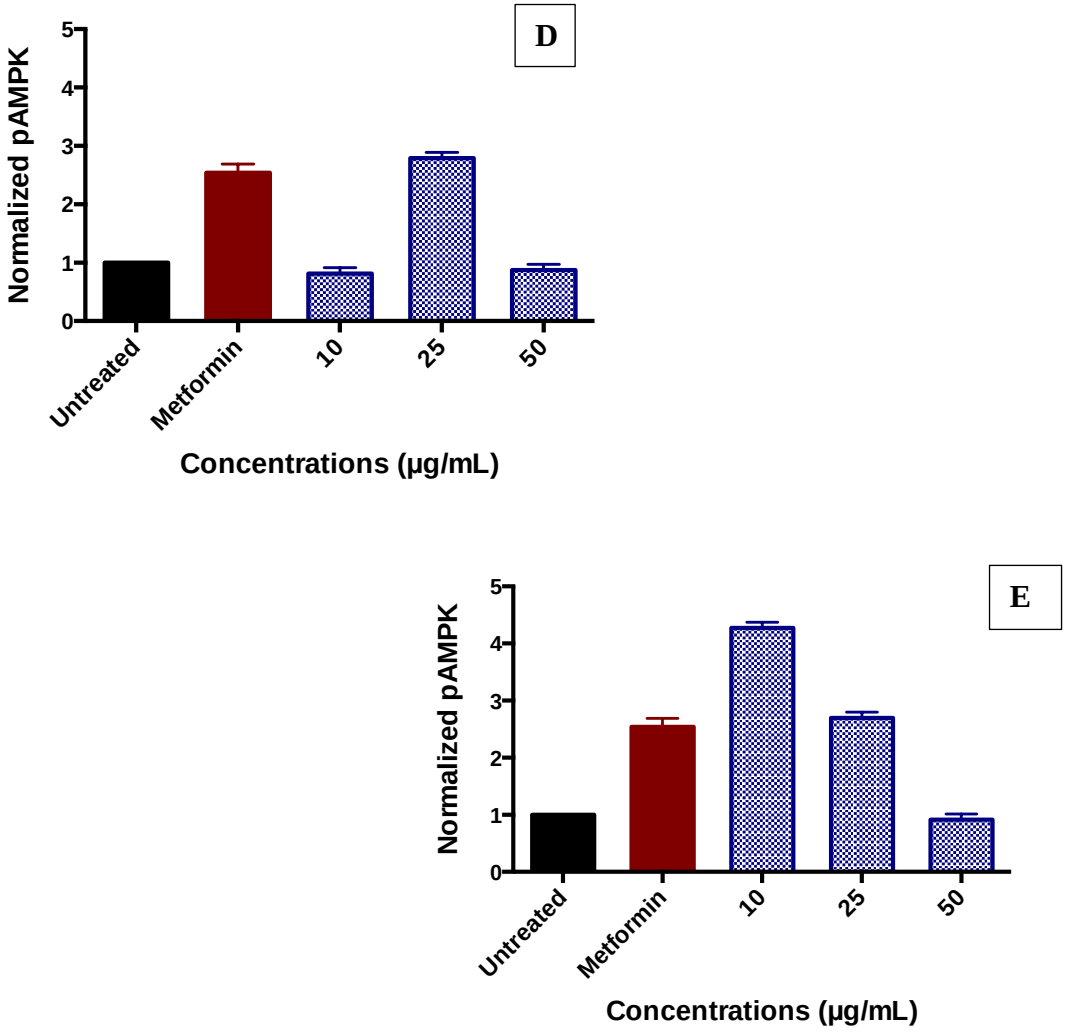

Figure 3.9: AMPK phosphorylation effect of the selected extracts in differentiated 3T3-L1 cells after 48 hours treatment with A) *C. limon* peel crude extract B) *C. limon* peel ethyl acetate fraction C) *C. limon* juice ethyl acetate fraction D) *B. integerrima* ethyl acetate fraction E) *T. bessarabicum* ethyl acetate fraction.*Results were expressed as ±SD of three different experiments carried out in triplicates, significantly compared to untreated control cells ($p<0.05$). *Measurements were performed by fluorescent ELISA, at 530/585nm.*Metformin was used in 100 µM concentration as positive control.

3.6.6 Determination of mRNA Expression Level by Quantitative Real Time Polymerase Chain Reaction

AMPK is a heterotrimeric enzyme with catalytic α1, α2, and regulatory β1, β2, γ1, γ2, γ3 subunits. The most expressed common isoforms are α1, β1 and γ1 and these are found in adipose tissue. α2, β2, γ2, and γ3 subunits are found in skeletal and cardiac muscles. Our selected extracts which were found to be effective on AMPK phosphorylation, were searched for evaluation on mRNA expression of AMPK. For this purpose, *mus musculus* genes encoding AMPK-α1 (Prkaa1), AMPK-β1 (Prkab1), and AMPK-γ1 (Prkag1) were studied by qRT-PCR and the results were given in **Figure 3.10**.

Most of the treated extracts increased Prkaa1 gene transcription significantly, except *T. bessarabicu*m ethyl acetate fraction. As a result, ethyl acetate extracts of *C. limon* peel (10 µg/mL) and *B. integerrima* (25 µg/mL) showed an increase in the gene expression of catalytic alpha subunit Prkaa1.

The ethyl acetate extract of *T. bessarabicum* had been found very effective, at almost 4 times of the control on AMPK enzyme activity by post-translational mechanisms; however in mRNA expression, its effect was only as little as the control, on Prkaa1 gene.

Moreover, ethyl acetate fractions of *C. limon* peel (10 µg/mL) and *B. integerrima* (25 µg/mL) were also found to upregulate the gene expressions of regulatory subunits of AMPK; Prkab1 and Prkag1, in qRT-PCR studies.

Metformin, a synthetic derivative of guanide which is a natural product in *Galega officinalis* plant, has been used as an anti-diabetic drug. It decreases hepatic glucose production and also improves insulin sensitivity (Foretz, 2014).

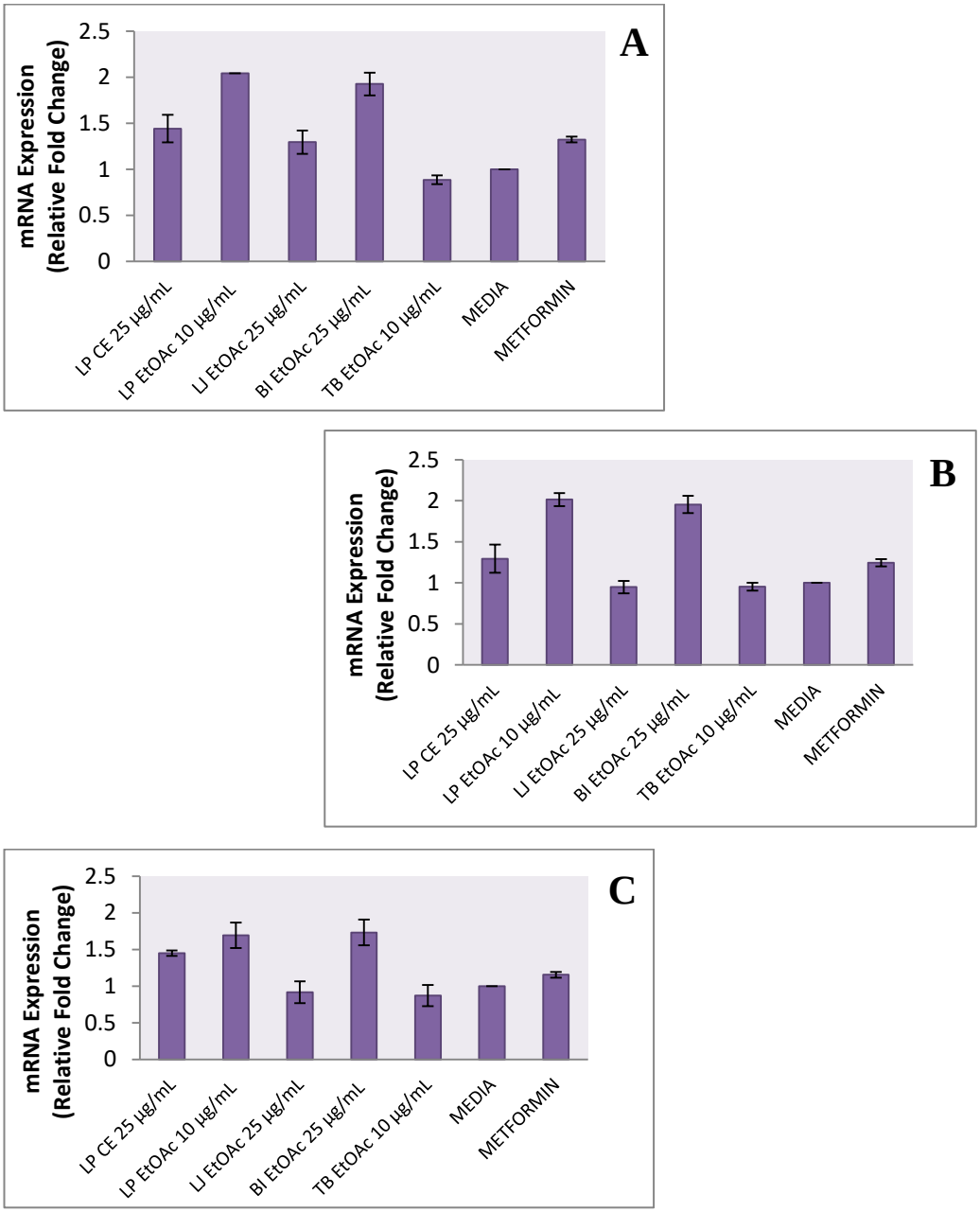

Figure 3.10: Effect of 48 hours treatment with samples on A) Prkaa1, B) Prkab1, C) Prkaag1 mRNA expression levels of 3T3-L1 cells. *qRT-PCR results were expressed as ± SD relative fold compared to untreated media control ($p<0.05$).

LP CE: *C. limon* peel crude extract, LP EtOAc: *C. limon* peel ethyl acetate fraction, LJ EtOAc: *C. limon* juice ethyl acetate fraction, BI EtOAc: *B. integerrima* ethyl acetate fraction, TB EtOAc: *T. bessarabicum* ethyl acetate fraction. *Metformin: 100 µM, positive control (AMPK activator) *All of the experiments were done as duplicates in two independent experiments.

As reported by Owen et al., it was shown that AMPK activation by metformin was not a result of direct activation; instead, metformin inhibited complex I mitochondrial respiratory chain, leading to an increase in the ratio of AMP:ATP (Owen, 2000).

In addition, lots of phytochemicals were shown to activate AMPK. Even though the differences in structures, they activate AMPK and show their beneficial effects on some diseases such as type 2 diabetes and metabolic syndrome. For example, resveratrol from red grapes, (Baur, 2006; Park, 2007); quercetin from many fruits, vegetables and grains (Ahn, 2008), berberine from *Coptis chinensis* (Lee, 2006), epigallocatechin gallate from green tea (Hwang, 2005), and curcumin from the plant *Curcuma longa* (Kim, 2009) were all shown to act as AMPK activators.

CHAPTER 4

CONCLUSION

This study was intended scientifically to develop a true dietary supplement for obesity. AMP-activated protein kinase (AMPK) has an important role in the energy expenditure. From this point of view, appropriate phytochemicals as the activators of AMPK were searched. For this reason, the selected plants *Citrus limon* (as peel and juice), *Berberis integerrima* and *Taraxacum bessarabicum* extracted and fractionated.

As a result, except for the hexane, all the fractionated extracts revealed a significant antioxidant capacity. Crude extracts and ethyl acetate fractionated extracts were considered quite effective as DPPH and ABTS radical scavengers and also were shown to have high phenolic and flavonoid contents. These crude and ethyl acetate extracts showing high radical scavenging capacity were carried out for the treatments on differentiated 3T3-L1 cells.

Crude extracts between the concentrations of 10-1000 μg/mL, and ethyl acetate fractions between the concentrations of 5-500 μg/mL did not show any cytotoxic effect on differentiated 3T3-L1 cell viability. Therefore, it was decided that treatments would be performed in these concentration range in enzyme activity and PCR assays.

The AMPK activation studies were carried out in the differentiated 3T3-L1 cells and was monitored via phosphorylation assay using fluorescence detected ELISA. 25 µg/mL of *C. limon* peel crude extract, 10 µg/mL of *C. limon* peel ethyl acetate fraction, 25 µg/mL of *C. limon* juice ethyl acetate, 25 µg/mL of *B. integerrima* ethyl acetate fraction, 10 µg/mL of *T. bessarabicum* ethyl acetate fraction were found effective on enzyme phosphorylation. Among the extracts ethyl acetate fraction of *T. bessarabicum* had been found very effective on AMPK enzyme activity, at almost 4 times of the control.

Subsequent to AMPK phosphorylation assays, further PCR experiments were performed to investigate the enzyme phosphorylation is coming whether from post-translational or pre-translational mechanisms in the cells. The ethyl acetate extracts of *C. limon* peel (10 µg/mL) and *B. integerrima* (25 µg/mL) upregulated catalytic alpha subunit Prkaa1 gene. Although the ethyl acetate extract of *T. bessarabicum* had been found almost as 4 times effective as control in AMPK phosphorylation; upregulation of Prkaa1 gene was not significantly induced compared to the control. Hereupon, it can be concluded that ethyl acetate fractionated extract of *T. bessarabicum* can possibly be activating AMPK through only the post-translational modifications. Ethyl acetate fractions of *C. limon* peel (10 µg/mL) and *B. integerrima* (25 µg/mL) were also found to upregulate the gene expressions of regulatory subunits of AMPK; Prkab1 and Prkag1.

By taking into consideration of post- and pre-translational AMPK modifications, ethyl acetate fractions *C. limon* peel, *B. integerrima* and *T. bessarabicum* were found to be effective AMPK activators compared to the untreated control. Those extracts that were effective AMPK activators can be considered as suitable for the preparation of dietary supplements against obesity.

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APPENDIX A

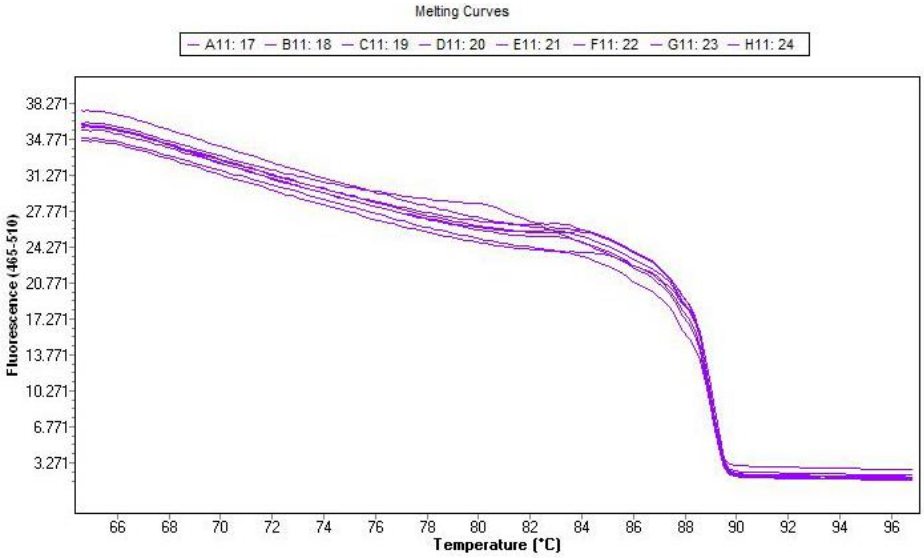


Figure A-1: Melting curves of beta-actin

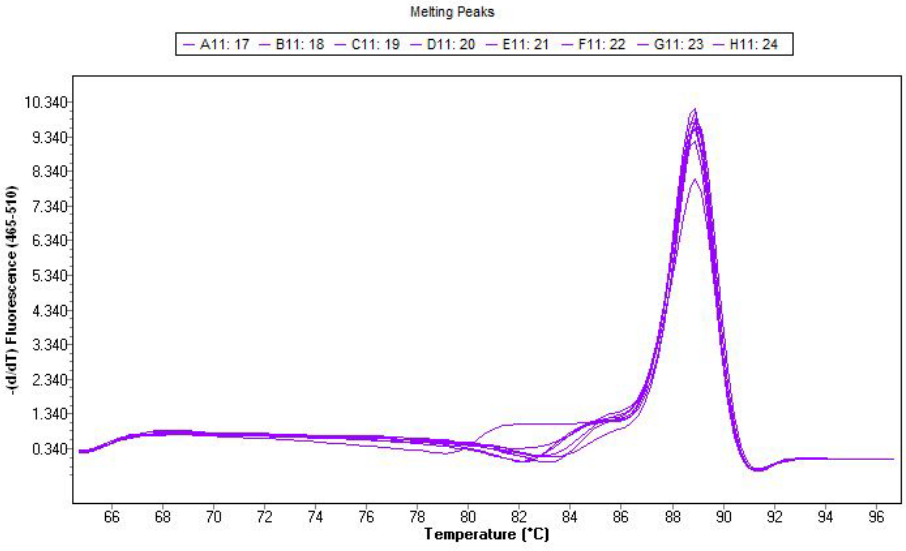


Figure A-2: Melting peaks of beta-actin

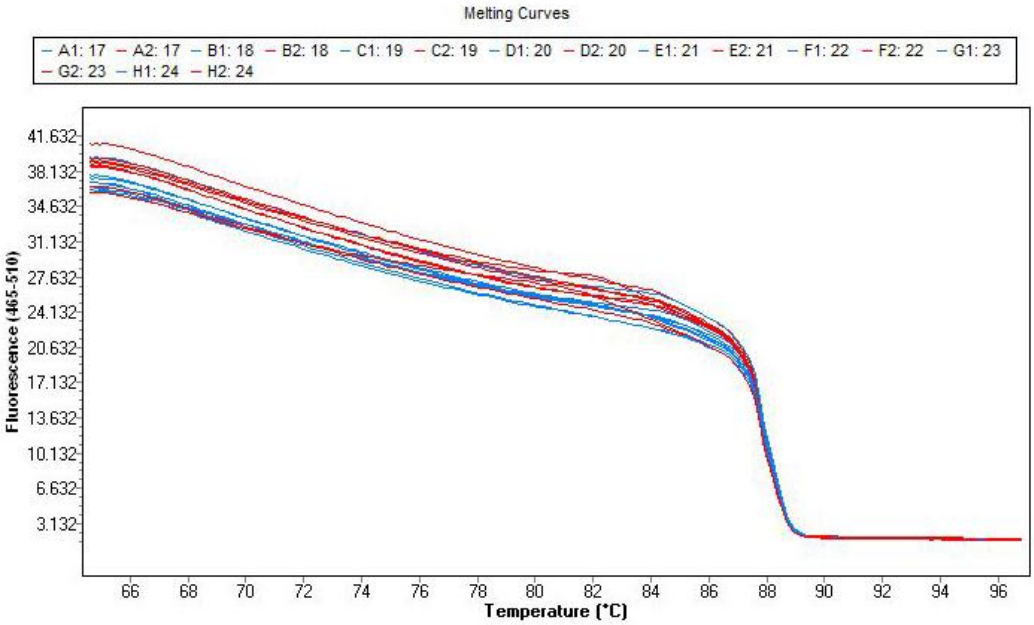
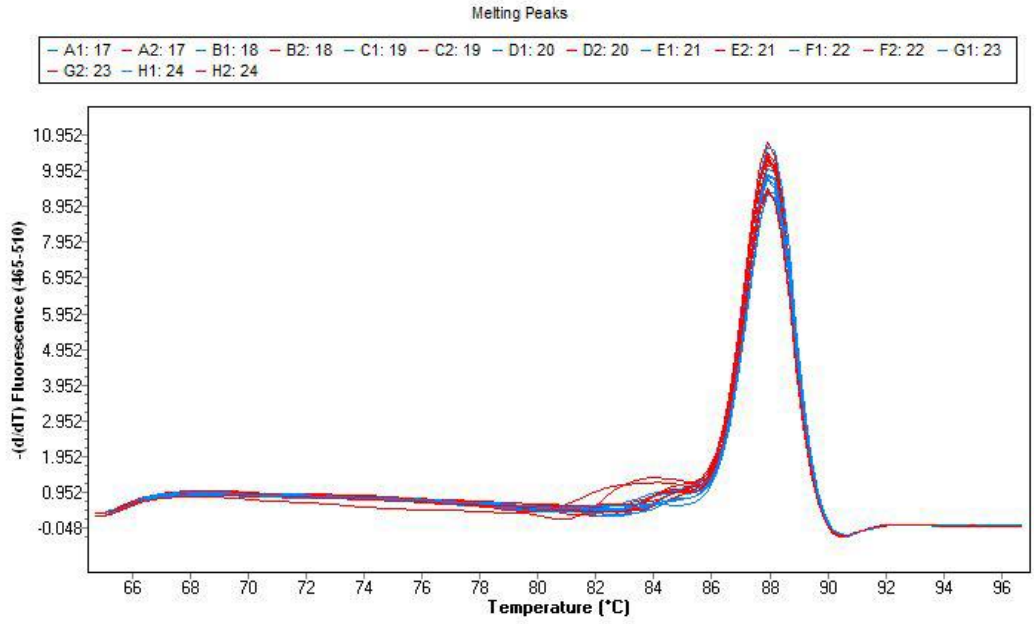


Figure A-3: Melting Curves of Prkaa1



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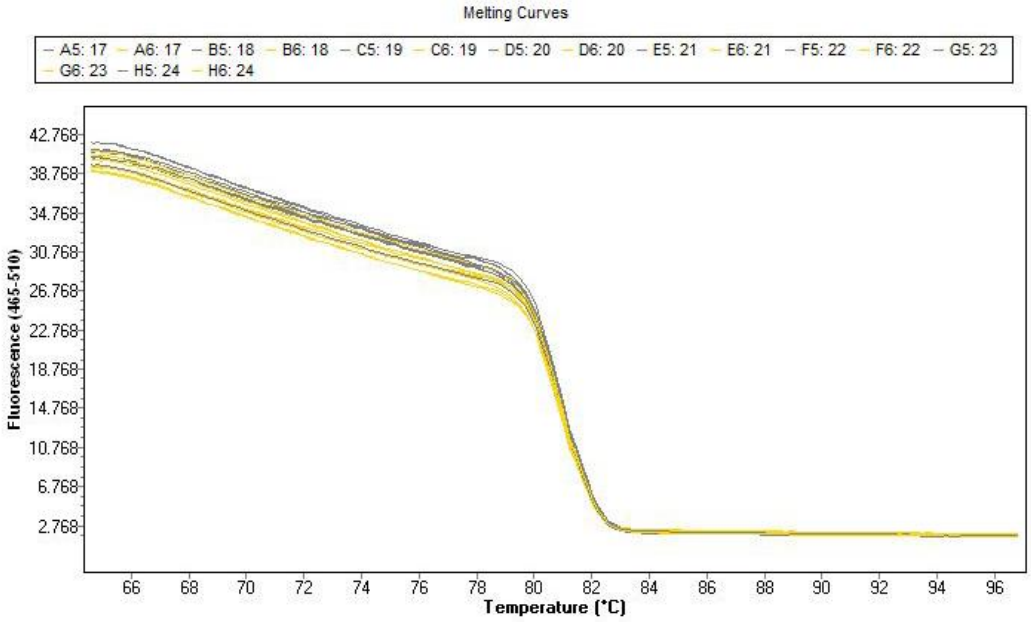

Figure A-5: Melting curves of Prkab1

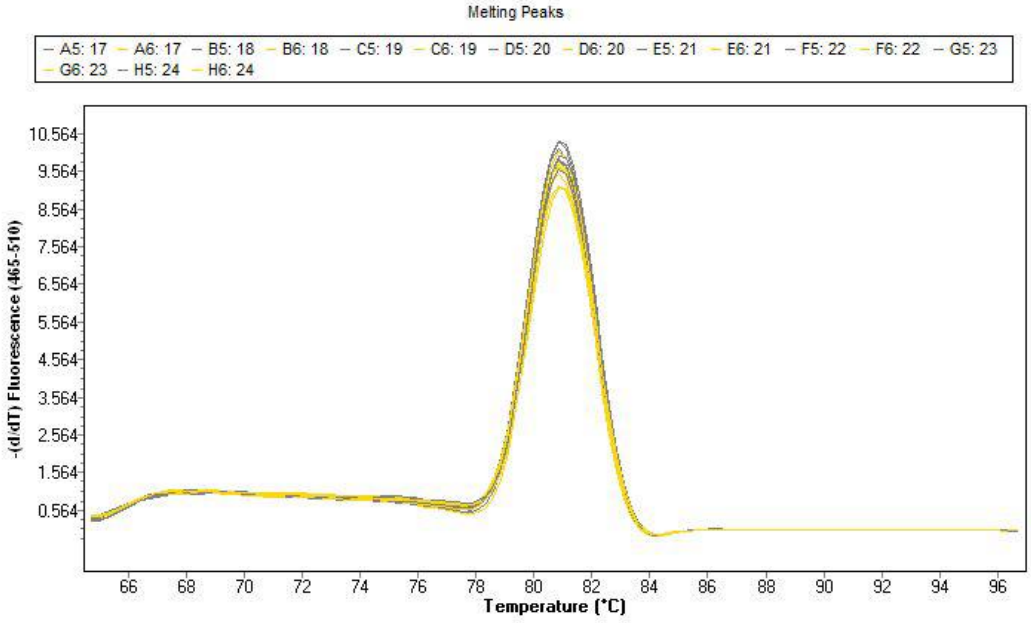

Figure A-6: Melting peaks of Prkab1

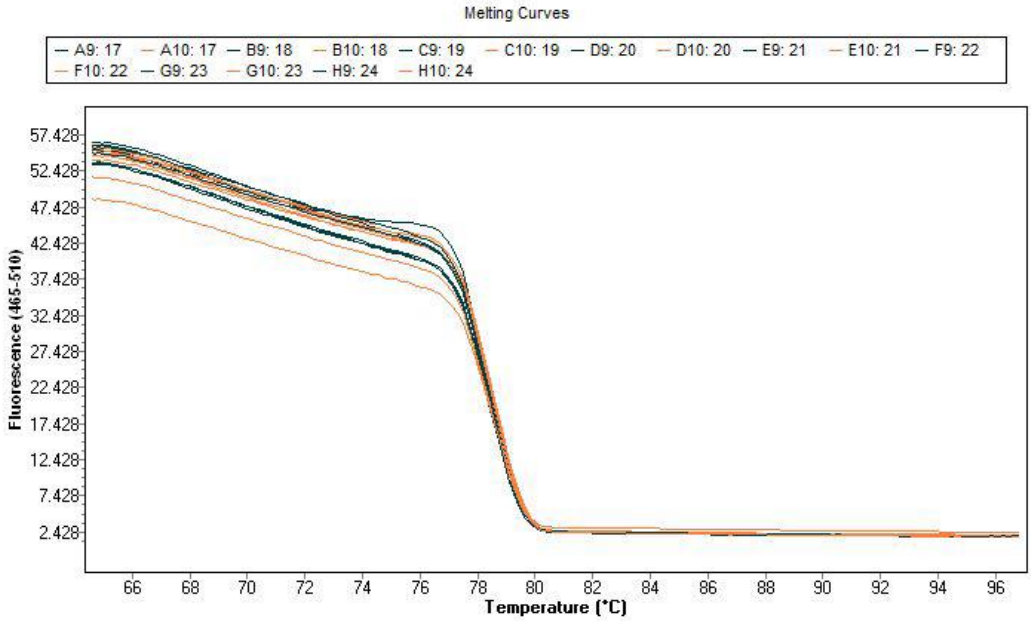

Figure A-7: Melting curves of Prkag1

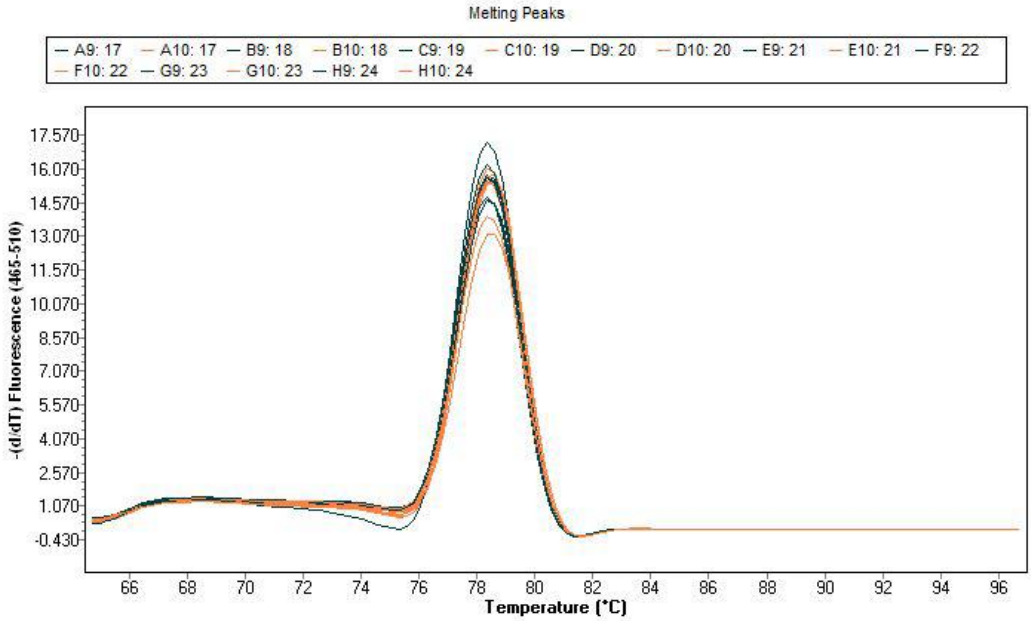

Figure A-8: Melting peaks of Prkag1

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Y. Kumbet, N. Çoruh. Antioxidant Capacity of Daily Consumed Pomegranate Fruit Juice and Its Antimicrobial Effect on *Proteus mirabilis*. Turk J Biochem, 2013; 38 (S1).

A. Aksoy , S. Sahin , **Y. Kumbet** and N. Coruh. Anti-oxidant capacity of *Aesculus hippocastanum* fruit parts as an anti-aging agent. FEBS Journal 280 (Suppl. 1) (2013) 3–617.

N. Ozdogan , S. Sahin , A. Aksoy , **Y. Kumbet** and N. Coruh. An investigation on the antioxidant activities and total phenolics content of *Calendula officinalis*. FEBS Journal 279 (Suppl. 1) (2012) 52–576.

N. Ozdogan, S.Sahin, **Y. Kumbet**, A.Aksoy, N.Coruh. Investigation of Total Phenolic and Flavonoid Content of *Centaurea cyanus* Extract And Its Free Radical Scavenging Capacity. Turk J Biochem, 2012; 37 (S1)

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•"Regulation of Metabolism and Metabolic Disorders" Graduate Summer School, Ege University and Karadeniz Technical University; Trabzon, 29 June-06 July 2008.

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