THE EFFECTS OF TRITICUM AESTIVUM LIN (WHEATGRASS) AND MENTHA PIPERITA (PEPPERMINT) DERIVED EXOSOMES ON ADIPOGENIC DIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS

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ABSTRACT

THE EFFECTS OF TRITICUM AESTIVUM LIN (WHEATGRASS) AND MENTHA PIPERITA (PEPPERMINT) DERIVED EXOSOMES ON ADIPOGENIC DIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS

In living organisms, when food intake is excessive, this excess energy must be stored, and multicellular organisms have specialized cells or organs to store this excess energy as lipids. The enzymes, hormones, growth factors, and cytokines secreted by adipose tissue play a vital role in the regulation of biological activities and energy metabolism in animals. Adipogenesis is defined as the transformation of preadipocytes into mature fat cells. In this study, adipogenesis was done by using human-derived adipose stem cells ordered from ATCC, and adipogenesis differentiation of these cells. In order to see whether they play a role in cellular fat breakdown, the exosomes isolated from *Triticum aestivum Lin* and *Mentha piperita* were characterized by size analysis on the NTA device. In order to determine whether exosomes have a toxic effect on human adipose stem cells, the MTS cytotoxicity experiment was carried out over six doses, and as a result, the experiments were continued by choosing a optimum dose. The optimum dose effect of exosomes from peppermint and wheatgrass on adipogenically differentiated cells were observed with Oil-Red-O staining. Adipogenic markers and their expressions in obesity markers were examined in the cells treated with mint exosome, in addition to these, the results were supported by ELISA and immunocytochemistry experiments. In line with these results, it was observed that peppermint exosome upregulated adipogenesis and induced obesity in cells treated with it. In addition, in cells treated with wheatgrass juice exome, it was observed that it downregulated adipogenesis and played a supportive role in the destruction of intracellular fat, as seen in the result of Oil-Red O staining. The findings of this study are encouraging for further research into the possible positive impacts of plant exosomes on adipose biology. In this sense, our findings guide for future research into plant exosome-based chemicals for the prevention and treatment of obesity, a modern pandemic with a strong relationship to comorbidities that is common globally.

ÖZET

TRITICUM AESTIVUM LIN (BUĞDAY ÇİMİ) VE *MENTHA PİPERİTA* (NANE) KAYNAKLI EKZOMLARIN İNSAN ADİPOZ KÖK HÜCRELERİNİN ADİPOJENİK FARKLILAŞMASI ÜZERİNE ETKİLERİ

Yaşayan organizmalarda gıda alımı fazla yapıldığında bu fazla enerjinin depolanması gerekir ve memelilerde yağların depolandığı adipoz dokusu görev almaktadır. Biyolojik fonksiyonların ve enerji metabolizmasının düzenlenmesinde adipoz dokusu salgıladığı enzim, hormonlar, büyüme faktörleri ve sitokinler ile önemli bir rol oynamaktadır. Adipogenezis, preadipositlerin olgun yağ hücrelerine dönüşmesi olarak tanımlanmaktadır. Bu çalışmada ATCC'den sipariş edilen insan kaynaklı adipoz kök hücreleriyle adipogenez farklılaşması yapılarak hücresel yağ modellemesi oluşturuldu. Hücresel yağ yıkımında rol oynayıp oynamadıklarını görmek için *Triticum aestivum Lin* ve *Mentha piperita* sularından izole edilmiş eksozomların NTA cihazında boyut analizi tekniği kullanılarak karakterizasyonları yapılmıştır. İnsan kaynaklı adipoz kök hücrelerinin üzerinde eksozomların toksik etkinin olup olmadığını tespit etmek için MTS sitotoksisite deneyi gerçekleştirilerek, sonucunda 100 µg/ml dozu seçilerek deneylere devam edilmiştir. Oil-Red O boyaması ile nane ve buğday çimi suyundan elde edilen eksozomların adipojenik olarak farklılaşmış hücreler üzerindeki etkileri gözlemlenmiştir. Nane eksozomu ile tedavi uygulanan hücrelerde adipojenik markerlara ve obezite markerlarında ki ekspresyonlarına bakılmış, bunlara ek olarak ELİSA ve immünositokimya deneyleriyle sonuçlar desteklenmiştir. Bu sonuçlar doğrultusunda, nane eksozomu ile tedavi uygulanan hücrelerde adipogenezi upregule ettiği ve obeziteyi indüklediği gözlemlenmiştir. Bunun yanı sıra, buğday çimi suyu eksozumu ile muamele edilen hücrelerde ise bu deneyler sonucunda adipogenezi downregule ederek, Oil-Red O boyaması sonucunda da görüldüğü gibi hücre içindeki yağ yıkımında destekleyici bir rol oynadığı gözlemlenmiştir. Bu çalışmanın sonuçları, bitki eksozomlarının adipoz biyolojisi üzerindeki potansiyel yararlı etkileriyle alakalı daha fazla araştırma yapılması için destekleyicidir. Bu bağlamda, bu sonuçlar, dünya çapında baskın olan ve tip 2 diyabet, hipertansiyon, kardiyovasküler hastalık, kanser dahil olmak üzere komorbiditelerle güçlü bir ilişki içinde bulunan modern çağın pandemisi olan obezitenin önlenmesi ve tedavisinde bitki eksozom bazlı bileşiklerin değerlendirilmesinin yolunu açmaktadır.

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LIST OF SYMBOLS/ABBREVIATIONS

aP2	Adipocyte Lipid-Binding Protein
C/EBP	CCAAT-enhancer-binding proteins
DEX	Dextran
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's PhosphateBuffered Saline
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
hADSC	Human Adipose-Derived Stem Cell
LPL	Lipoprotein Lipase
mL	mililiter
MISEV	Minimal information for studies of extracellular vesicles
PEG	Polyethylene Glycol
PPAR	Peroxisome proliferator-activated receptor
PSA	Penicillin/Streptomycin/Amphotericin
SREBP-1	Sterol Regulatory Element Protein1c
UCP	Mitochondrial Uncoupling Protein
USA	The United States of America

1. INTRODUCTION

Appropriate and balanced nutrition is fundamental for protecting and improving the health of individuals and increasing their quality of life. Its importance is emphasized in terms of preventing diseases and protecting health [1].

On one hand the health problems caused by malnutrition can be prevented with improved economic and technological conditions of living, on the other hand problems related to excessive nutrition are on the rise as being reported with alarming statistics in regards to obesity and metabolic diseases in several epidemiologic studies [2]. With changes imposed on contemporary life styles, both people's eating habits and mobility change with the developing technology, in a way such that daily caloric intake increases while frequency of movement decreases come together. As a result, the prevalence of obesity in our nation and throughout the world is rising [3].

Obesity is one of the most critical health issues in both industrialized and developing nations, and it is associated with a wide range of other diseases. Obesity is a condition that happens when the energy received from food exceeds the energy spent, causing the body weight to grow over the desirable level due to a rise in the ratio of body fat mass to lean body mass [4].

Like in the case of obesity, other metabolic diseases occur with excessive increase in body weight. Obesity has now been accepted as a pandemic disease by the World Health Organization and this has become an important problem that concerns the whole world. For this reason, there are many variables that contribute to obesity, and it is crucial to identify them in order to address the related health issues and take appropriate precautions [5].

1.1. EPIDEMIOLOGY OF OBESITY

Although obesity occurs as a result of an imbalance between caloric intake and use, various factors are effective in its etiology [6]. Some of these factors are; genetic factors, age, nutritional habits, physical activity, psychological factors and socioeconomic cultural level. The increment in obesity rates can be attributed to a variety of factors, including a decline in physical activity levels around the world and in our country, a preference for a diet high in

saturated fats and salt, as well as an increase in time spent in front of a television or computer [7,8].

Obesity is a very common health problem in almost all societies and is increasingly becoming a global pandemic. According to WHO determinations; Obesity worldwide has doubled since 1980. Estimates suggest that 1.4 billion adults were overweight, 200 million men and 300 million women were obese in 2008. When these numbers are expressed as prevalence; as of 2008, the incidence of overweight and obese individuals in the world is 35%, and 11% worldwide respectively [9].

It is accepted that 25% of developed societies are obese, 25% are overweight, 25% are normal weight, but genetically predisposed to obesity. This last group includes individuals who can maintain their weight with continuous diet and exercise efforts, and who can easily gain weight and switch to the overweight or obese class if they fall out of their dietary and physical activity related habits. In these people, metabolic mechanisms work similarly to those seen in obese people. Therefore, the definition of "metabolic obese" has been used to describe this group of individuals in recent years. On the other hand, researchers report that there is a group that is classified as overweight or even slightly obese, but metabolically completely normal, and the definition of "healthy obese" should be used for them [10].

1.2. DEFINITION AND IMPORTANCE OF OBESITY

The World Health Organization presently classifies obesity as a pandemic disease. Obesity is defined as a chronic condition when the energy of the food ingested exceeds the energy exhaled [11].

The main mechanism in the formation of obesity is positive energy balance, that is, as the calories consumed are less than the calories taken with food, the calories accumulated in the body are converted into adipose tissue and stored. Obesity, a major health concern that results from an excessive accumulation in adipose tissue and can lead to a variety of illnesses and even death, is a disease with severe morbidity and mortality that affects all organs and body systems [12].

1.3. ETIOLOGY OF OBESITY

Obesity is a chronic condition and there are many factors such as genetic, metabolic, hormonal, psychological, socioeconomic factors, nutrition and physical activity level [13].

The daily amount of energy required for the body is 1100-1300 k.cal, and there are 3 major components in the expenditure of this energy: resting metabolic level, thermogenesis due to physical activity and thermic response due to nutrition [14]. These components are the most important energy components on the presence of adipose tissue [15]. The activation of adipose tissue lipoprotein lipase is another regulatory mechanism controlled by adipose tissue (ALPL). This enzyme is formed in adipocytes and is in constant contact with endothelial cells as it is released into the extracellular space [16]. At this localization, ALPL reacts with circulating triglyceride-rich lipoproteins to hydrolyze triglyceride into fatty acids. The resulting fatty acids are taken up by adipocytes, converted into triglycerides and stored. This shows that ALPL causes excess fat to be stored in adipose tissue with the contribution of ALPL, especially in those fed with foods rich in saturated fats [17,18].

Most of the plasma cholesterol is transported by low-density lipoproteins (LDL). However, especially during fasting periods, very low-density lipoproteins (VLDL) carry a large proportion of circulating triglycerides. Obesity also has a significant effect on VLDL metabolism. Hypertriglyceridemia is common in obese patients and correlates with the severity of obesity. This increased triglyceride level is due to the increase in hepatic VLDL production [19].

Factors affecting the formation of obesity are as follows;

Age; The use of energy from food is determined by the level of physical activity, the thermal power of the diet, and the basal metabolic rate. 12% of the energy consumption of a person in twenty-four hours occurs with physical activity, 15% with thermic effect and 73% with basal metabolism. Therefore, the incidence of obesity is more common in women with advancing age [20].

Gender; In many countries, it is observed that the proportion of fat in women is higher than in men. After puberty, girls gain more weight than boys, and because of weight gain during pregnancy and childbirth, obesity is more pervasive in women than in boys. While the adipose tissue is 20-25% in 18-year-old girls, this rate is around 15-18% in boys [21].

Sociocultural factors; The diet of families, the eating habits of societies, the living environment, genetic structure, work conditions, education level affect the frequency of obesity. The amount of food taken at meals, the number of meals, excessive consumption of carbohydrates cause excessive calorie intake. Economic conditions, scarcity of playgrounds,

spending hours in front of the computer reduce physical activity and lead to weight gain. Obesity, especially among women, is more common at lower socioeconomic levels [22].

Socioeconomic factors; According to research, while the frequency of obesity grows in direct proportion to an individual's wealth, the incidence of obesity decreases with an individual's degree of education [23].

Physical activity; Physical exercise is an important factor of the energy cycle since it helps to maintain body weight and energy balance. Being inactive is one of the most important causes of obesity. In modern societies, carrying out work with less energy, spending more time in front of the television causes the body to store the energy that it cannot use as fat. In a study, it was determined that physical inactivity was responsible for 67.5% of obesity etiology. According to epidemiological studies, obesity in men is mostly seen in those who live a sedentary life. In recent studies, it has been determined that watching television more than 2 hours a day in childhood and adolescence causes obesity, cardiovascular diseases, and increased serum cholesterol in early adulthood [24].

Genetic factors; Genetic factors are considered to have a substantial influence on the prevalence of obesity. Therefore, in the study conducted on twins, it was determined that the twins who were adopted and raised with their own families were obese despite being brought up in different environmental factors. It has been determined that the fat ratio of children is similar to the fat ratio of their families, and the probability of obese children to be obese is 2-3 times higher than that of non-obese individuals [25].

1.4. GENETICS OF OBESITY

Studies have demonstrated that genetics have a role in obesity, and it has been determined that genes directly cause obesity in diseases such as Prader-Willi and Bardet-Biedl syndrome [26]. The future cannot always be predicted by genes, though. Genes and habits must work together to cause obesity. Exogenous variables such as excessive food intake and insufficient physical exercise can enhance a person's risk of obesity when more than one gene is involved. In addition to these, other contributing factors include some diseases such as Cushing's disease and polycystic ovarian syndrome, as well as the use of certain drugs such as steroids and antidepressants. However, other high-calorie diets, lack of physical activity, age, socio-economic status, race, smoking cessation, and family history are also among other risk factors [27].

1.4.1. Genetics of Obesity in Humans

Although it is seen that rare monogenic forms of obesity in humans are not responsible for most of the cases; Common forms of obesity are thought to be due to complex interactions between genes and the environment. Also, the mechanisms by which genes can affect obesity include body fat pattern, appetite regulation, and other metabolic and signaling pathways[28].

Familial agglomeration and twin studies suggest that the higher incidence of comorbidity in identical twins compared to fraternal twins is evidence supporting the genetic effect of obesity in humans [29].

Mutations in some of the following genes are known to cause monogenic forms of human obesity, some of which are: LEP, LEPR, POMC, MC4-R, GPR24 , NROB2. Mutations in the LEP gene are a notable example of human monogenic obesity, and it has been shown that leptin deficiency and therefore the mutation that causes obesity can be reversed by leptin hormone therapy [30]. A similar situation has been supported by obese (Ob) and diabetic (Db) animal models.

In addition, studies have found that there is a correlation between many loci and candidate genes and mutations in these genes and a polygenic, multifactorial transmission pattern between overweight or obesity. These loci include POMC, UCP1, LEPR, LDL, SIM1, PPARG, LIPE, LEP, MC4R, TNF, UCP2, and UCP3. Melanocortin signaling pathway, which includes some of these genes, which has been revealed in many studies and still continues, has come to the fore, and it has been determined that mutations in many genes and related genes that play a role in this signaling pathway are associated with obesity and related phenotypes [31].

1.4.2. Genetic Predispositon to Obesity

It is known that obesity shows familial accumulation. However, with the exception of some rare diseases accompanied by obesity, the majority of obese patients do not show a complete Mendelian inheritance. The amount of obesity's heredity was assessed through twin, adoption, and family analyses. In body mass index-based investigations, it was established that identical twins and fraternal twins, as well as identical twins raised separately, accumulated 70% BMI variance, indicating a high amount of heredity. Adoption studies

have demonstrated a heritability of 30% or less. In most family studies, modest heritability was seen between twin and adoption studies [32]. In various research, the heritability of BMI has been estimated between 25 and 40%.

A statistical method called the "lambda coefficient" can be used to figure out how likely someone is to be obese if they have an obese or overweight first-degree relative. This technique compares the risk of obesity in the biological population to the population-wide risk of obesity. According to a research on this issue, the risk ratios for 2349 first-degree relatives of 840 obese individuals were twice as high as the risk ratios for the general population. Furthermore, the risk rises with the severity of the individual's obesity. As a result, the risk of extreme obesity is eight times greater in families with extremely obese members. According to a Canadian research of 15245 persons, the family risk of obesity is five times higher among relatives than in the overall Canadian population [32].

1.4.3. Obesity and Adipogenesis

The increase in volume and number of adipocytes, which draws attention as the main factor of obesity; known as adipocyte differentiation. Adipogenesis, on the other hand, is a multifaceted process involving the transformation of progenitor adipocytes into mature adipocytes, and it constitutes the process of proliferation, differentiation, intracellular lipid storage and expression of related genes of progenitor adipocytes.

The gene expression program during adipogenesis is extremely intricate and well-regulated and additionally, the genes which are CCAAT/enhancer binding proteins (C/EBP- α) and Peroxisome proliferator-activated receptor (PPAR) are critical early regulators of this process [33].

One of the earliest families of transcription factors to have their importance in adipocyte development recognized was the C/EBP family. Tissues like the liver express isoforms of C/EBP, which metabolize lipid and cholesterol-related substances [34]. C/EBP-α does not directly cause differentiation, although it is expressed before the transcription of most adipocyte-specific genes. All three isoforms of C/EBP-α, C/EBP-δ and C/EBP-β express themselves in a sequential and coordinated fashion throughout adipocyte formation. C/EBP-α is upregulated during differentiating from progenitor adipocytes to adipocytes of the β and δ subtypes [35].

The PPARs interact to the retinoid X receptor (RXR) to create heterodimers, which then control transcription. PPARγ is one of the most important adipocyte-specific genes with a critical role in promoting growth and initiating adipogenesis as well as regulating adipocyte differentiation. PPARγ induces adipocytes during differentiation from progenitor adipocytes to mature adipocytes and underlies this process. In the absence of its effectiveness, differentiation cannot be made [36].

Co-expression of C/EBP-β and C/EBP-δ directly induces PPARγ expression. C/EBP-α and PPARγ levels rise dramatically during adipocyte development. This implies that increasing C/EBP-β above a specific threshold stimulates PPARγ expression and C/EBP-α ligand stimulation of PPARγ results in complete adipocyte differentiation [35].

1.5. CURRENT TREATMENT METHODS IN OBESITY

Dietary and behavioral therapy, especially the recommendation of a hypocaloric diet, has formed the basis of obesity treatment in the last 40 years. Unfortunately, calorie restriction alone is often ineffective. It is known that many dietary approaches lead to weight loss in the first period, but weight loss cannot be maintained in the long term.

The application of very low calorie diets (800 calories per day) in people with a body mass index above 30 can provide a loss of 2 kg per week and 20 kg in 4 months. However, not all of these lost weights come from adipose tissue. An estimated 16-20% is lost from supporting tissue, which is very beneficial for the body. Maintaining weight loss after starting this diet has been shown to be difficult, especially without patient education. This may be due to a decrease in energy expenditure due to energy deprivation. Behavioral consequences of semi-starvation (fatigue, weakness, loss of motivation) also prevent permanent weight loss. It is not possible for many people to maintain such a calorie restriction in the stress of life.

Better-structured programs that combine diet (restriction of 1200-1800 calories per day) with exercise and behavioral therapy may be more successful, but even this success is debated. Such programs can provide a loss of 9-14 kg in 5-6 months and the failure is as low as 20-24%.

Exercise can be the most effective solution for weight loss. Meta-analyses evaluated the effect of exercise on the maintenance of free fat mass during diet-induced weight loss. While 28% of the weight loss was free fat mass in people whose diet was only included in the

program, only 13% of the weight loss was from free fat mass in those who exercised in addition to the diet. When combined with reduced energy intake, exercise results in more weight loss than diet alone and preserves muscle mass. Most importantly, exercise ensures that weight loss is maintained for a longer period of time. For these reasons, exercise should be included in programs aimed at reducing body weight.

Surgical treatment of morbid obesity has been used as an option since the development of the jejunoileal bypass in the 1950s. Although there are a wide variety of surgical methods, all of them have risks of serious complications. Intraoperative complications, anesthesia risks and postoperative problems (sepsis, bacterial contamination of the cecum, fatty liver development and cirrhosis) can be counted.

The most frequently recommended drugs in the treatment of obesity have been appetite suppressing agents. Various classes of drugs have been used, such as serotonin agonists, sympathomimetics, and more recently leptin.

The endocannabinoid system, which is a neural regulatory system that controls food intake, has recently attracted a lot of attention. In addition to their effects on appetite, endocannabinoids have many other effects, such as alterations of hepatic fatty acid synthesis. With the discovery of the cannabinoid receptor CB1, to which tetrahydrocannabinol, the psychoactive component of marijuana, specifically binds, agents that block this receptor have been developed. Early clinical trials reported reduced food intake and 5-8% loss in body weight. Future research in this area is promising. Several central neurotransmitter pathways are currently under investigation (with clinical trials) in the treatment of obesity. Although none of them has been approved yet, therapeutic researches on these pathways are carried out very intensively and may change the treatment of patients in the near future.

Thermogenic agents constitute a theoretically interesting approach, given that low resting energy expenditure is indicative of the development of obesity and that weight gain has effects that increase Total Energy Consumption and fat oxidation. Various classes of thermogenic agents are being tested. Although thyroid hormone is catabolic, it is not useful in the treatment of obesity. Administration of recombinant leptin (ob gene product) increases total energy expenditure and activates the sympathetic nervous system as well as reduces food intake in ob/ob mice. The net effect of leptin administration in the animal model without

leptin is a reduction in adipocyte count. Similar results have been obtained in clinical trials with recombinant leptin in humans.

1.6. STEM CELLS

Stem cells are defined as cells that can divide indefinitely in the body of living organisms, self-renew, do not have a specific tissue identity, maintain their undifferentiated phenotype until the suitable message is received, and can differentiate according to the body's needs and transform into cells of other tissues. While stem cells are renewing themselves by proliferating, they are employed to regenerate by maintaining their quantity, which generates the primary cells that compose all of our tissues and organs [37].

One of the most distinguishing characteristics of stem cells and progenitor cells is that stem cells not only generate the cell that will become a progenitor cell, but also construct a backup copy of themselves during division. As a result of asymmetric cell division, this process guarantees the stem cell pool remains stable throughout life. The extracellular microenvironment of stem cells is responsible for their asymmetries. Components of the microenvironment's extracellular matrix, surrounding cells, and the proper proteins maintain the stem cell's condition and number under control [38]. As stem cells move away from the microenvironment, they rapidly lose their ability to renew themselves. While the proteins released by these cells serve as biochemical factors, the fluidity of the microenvironment affects the stem cells as a physical determinant and influences their self-renewal activities. Many changes happen in both cellular and extracellular structures in the microenvironment throughout aging, injury, illness, and development, determining whether the stem cell is influenced favorably or adversely. The fact that stem cells' self-renewal mechanisms are in a delicate balance plays a vital part in the formation of cells that need to be replenished [39].

Studies in the stemcell biology and the development of cell culture techniques are making fast progress. As a result, stemcell implementations in the cure of many diseases, especially diseases and damage to the nervous system, metabolic diseases, organ failures, rheumatic diseases, heart diseases, bone diseases, and cancer, are expected to have positive results in the near future. Stem cells have become more important in recent years as a way to treat many diseases that require organ or tissue transplants but don't respond well to other treatments. These stem cells, which can make new copies of themselves and change into

different types of cells, are divided into three types based on how they divide and change [40].

1.6.1. Totipotent Stem Cells

The zygote, which is made when the sperm fertilizes the egg, is a totipotent cell that can do all the things that an adult organism needs to do. Even though a totipotent cell can make a living thing on its own, it can still do this for 4 days after it has been fertilized [41].

1.6.2. Pluripotent Stem Cells

The pluripotent cells generated from the inner cell mass of the blastocyst are known as embryonic stemcells. Three embryonic germ layers may be formed from these cells, and an adult's germ layers include around 220 distinct cell types. In regenerative medicine, embryonic stem cell research is a major focus because of its capacity to regenerate and convert into a diversity of different cell types [42].

1.6.3. Multipotent Stem Cells

Adult stem cells which is known as multipotent stem cells, are capable of transforming into any organ or tissue from which they are derived. These are undifferentiated cells that may repair injured or dying cells in mature tissues in adults. They are also referred to as somatic stem cells. Adults may not have the same quantity or potential for stem cells in every organ and tissue. These cells, for example, are present in relatively small quantities in the brain. As a result, when the brain is damaged, tissue or bone organ regeneration does not occur, and the damage is typically permanent. The stem cell group employed in therapy is made up of these multipotent stem cells [43].

i. Hematopoietic stem cells (HSC): Myeloid cells and lymphocytes are terminal cells created via differentiation as the progenitors of all blood cells. Adult bone marrow, hips, thighs, ribs, chest, and other bones contain hematopoietic stemcells [44].

ii. Mesenchymal stemcells (MSC): These stem cell types, which are present in bone marrow, cord blood, and adipose tissue, have an extremely high ability for self-renewal, differentiation, and, most importantly, tissue repair [45].

iii. Adipose Tissue StemCells (ASC): adipose tissue stem cells may be isolated from human adipose tissue using lipoaspirate and manual special procedures in a laboratory environment.

Human adipose stem cells have been proven in experiments to be capable of differentiating into cartilage, bone, fat, and muscle cells in the laboratory conditions [46].

1.7. ADIPOSE STEM CELLS

Although adipose tissue is not a useful tissue, recent research shows that it provides a great source of adult tissue. They can also differentiate rapidly into osteogenic cells and many other cell types. This brings to mind their use to overcome problems in different fields, especially in tissue engineering and large bone defects. A lot of work is being done to obtain bone marrow stem cells in tissue engineering. However, the bone marrow does not produce enough cells. Adipose tissue, on the other hand, allows for the safe collection of huge numbers of stem cells [47].

Adipose stem cells are multipotent cells produced by isolating adipose tissue (ASC). ASCs are acquired from healthy donors via a process known as liposuction, which involves suctioning subcutaneous adipose tissue cells from the body using a vacuum, and they exhibit characteristics such as fibroblast-like morphology, facile adherence, and differentiation into mesenchymal cell lines. It has been shown that pluripotent stem cells derived from adipose tissue have the same ability to develop as bone marrow stem cells. This condition has piqued the curiosity of numerous professions, including tissue engineering [48].

For multiple reasons, adipose-derived stem cells are able to be the most promising for cell treatment among the several kinds of mesenchymal stem cells (MSCs). It has been demonstrated that these cells, like other mesenchymal stem cells, may turn into myocytes and neurons, as well as osteogenic, chondrogenic, and adipogenic differentiation, in the tissues and organs for which they are suited [49].

There are two forms of adipose tissue, both of which create white adipocytes from mesenchymal stem cells (MSCs) that expand through the neuroectoderm and mesoderm. MSCs are distinguished by their capacity to stick to plastic, proliferate, and develop into osteocytes, chondrocytes, and adipocytes from mesenchymal stem cell lines. MSCs are found in adipose tissue, bone marrow, and other tissues' vascular stroma. There are several variables that promote or hinder MSC development in the adipocyte line. BMP-2 and 4 signaling, in particular, stimulate white adipocyte development. MSCs are directed to the adipocyte line by Wnt signaling, which then suppresses differentiation. Adipocyte phasing is likewise inhibited by Sonic Hegdhog signaling [50].

Brown adipocytes originate from the paraxial mesoderm and develop from the coprogenitor with skeletal muscle, which express Myf523. BMP-7 triggers the transformation of progenitor cells into adipocyte line by being induced by PRDM16 containing the PRD1-BF1-RIZ1 homologue and peroxisome proliferation activator receptor (PPAR)-gamma-coactivator-1α. PRDM16 is specific for brown adipocytes and they express the progenitors Myf5 with activation of the PPAR-γ transcriptional function [51].

Adipose tissue has recently gained importance due to its functional complexity and structure. Its main subcomponents, mature adipocytes and stromal vascular differentiation, have assumed many physiological roles. Adipose stem cells can be obtained by centrifugation directly from adipose tissue in the form of stromal vascular differences in tissue engineering or regenerative medicine, and they can be used without culturing, as well as by culturing. When cultured, proteomic sites are determined step by step. There are thousands of known genes and proteins whose transcription and secretomes need to be observed. By using very precise protein-determining tools and smart bioanalytical tools, studies are gained about proteomes [52].

1.8. EXOSOMES

Since billions of cells in multicellular organisms need to communicate in order to perform their functions simultaneously, intercellular communication, which continues throughout the life of living things, is of vital importance. This intercellular communication takes place by secreting chemical messengers against the nearest or farthest cells. Depending on the distance between cells, local communication and hormonal communication take place [53]. While regulators such as neurotransmitters and local regulators transmit messages to cells in short distances, hormones and extracellular vesicles are involved in long-distance intercellular communication. Extra vesicles are lipid-layered nanovesicles that produce cell-specific macromolecules, including as DNA, proteins, messenger RNA (mRNA), and microRNA (miRNA). All fluids released by the body, including serum, plasma, amniotic fluid, saliva, breast milk, and urine, can yield extracellular vesicles [54].

Exosomes are natural nano-vesicles that are released by cells to the extracellular environment and vary in size, generally between 20-100 nanometers. These vesicles, which are surrounded by a double-layered phospholipid membrane, contain various nucleic acid types and derivatives, lipids and proteins. The existence of these vesicles, which are located

outside the cell and surrounded by a membrane, has been known for about 50 years. However, the discovery of exosomes with the functions known today dates back to the 1980s [55]. When they were first discovered, it was thought that exosomes were only responsible for transporting waste from the cell out of the cell. However, with the discovery that exosomes are used to remove the transferrin receptor during the maturation of red blood cells, then their immunological role in B-cells, and finally the discovery that they contain various nucleic acid derivatives and their role in cancer metabolism, exosome studies have moved to very different dimensions. Exosomes have been detected in a wide range of human fluids, including breast milk, urine, saliva, perspiration, serum, and plasma, during the past several years [56].

1.8.1. Biogenesis and Structure of Exosomes

In exosome biogenesis, it first occurs with the differentiation of the early endosome, which is formed by budding by invaginating the main cell membrane. This process is controlled by ceramide and the ligand-receptor complex is taken from the cell membrane and transferred into the resulting endosome [57]. The early endosomes formed bud inward to form numerous intraluminal vesicles (ILVs). The development of these intraluminal vesicles involves tetraspanins such as CD9 and CD63 (Figure 1.1.). The early endosomes that embrace intraluminal vesicles are known as multivesicular bodies [58]. Multivesicular bodies are transformed into dissimilar end products according to their target and content. Multivesicular bodies follow two paths, one of which can be removed by going to the lysosome via the trans-Golgi network. The second way is to go to the plasma membrane and take the name of exosome by exocytosis and secrete out of the cell [59].

Four subunits make up the ESCRT proteins on the surface of intraluminal vesicles, which have a role in the production of exosomes. ESCRT-0 contributes to their identification by amassing uniquitin proteins. While ESCRT-1 and ESCRT-2 proteins are accountable for membrane budding, ESCRT-3 is involved in the separation of this membrane. This method involves the development of a complex containing Tsg101, Alix, ESCRT-1, and ESCRT-3 in the membrane of intraluminal vesicles [60].

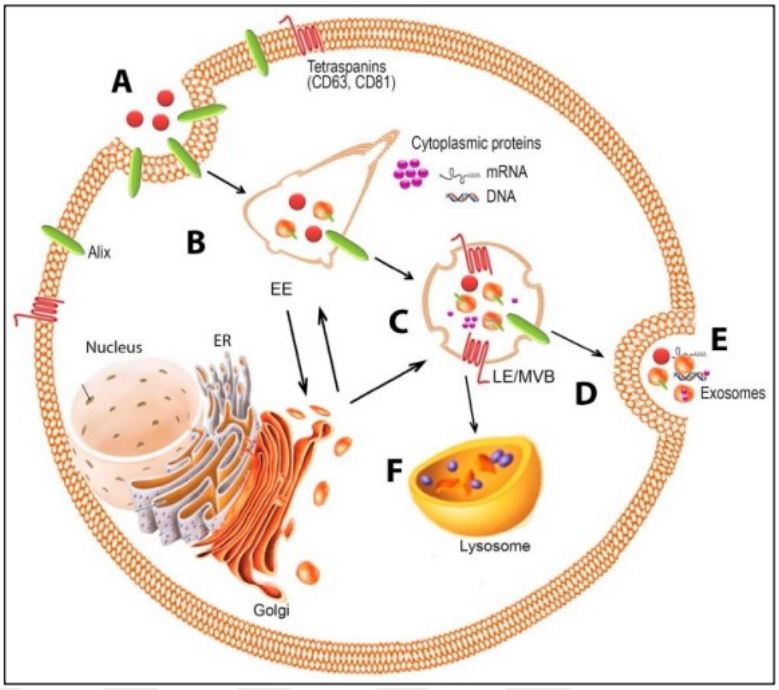

Figure 1.1. Exosome biogenesis and release from a cellular perspective [57]

Proteins belonging to the Ras-associated binding protein family have a role in exosome release from the trans-golgi network. These proteins are involved in many cell types' secretion of exosomes; they allow the fusion of the plasma membrane with MVC's limiting membrane [58]. Exosome secretion and endosome advancement are both aided by members of the Rab family of proteins. Furthermore, Rab7 is a protein involved in the direction of MVC to lysomy, while Rab4 and Rab5 are involved in the conversion of early endosomes into MVC. This process is provided by the direct or indirect action of the SNARE (soluble N– ethylmalimide-sensitive fusion protein-linked protein complex) protein family. In addition to these, the membrane proteins found in the membrane of the MVC and the intraluminal vesicles formed within it also function in the secretion of exosomes (Figure 1.2.) [61].

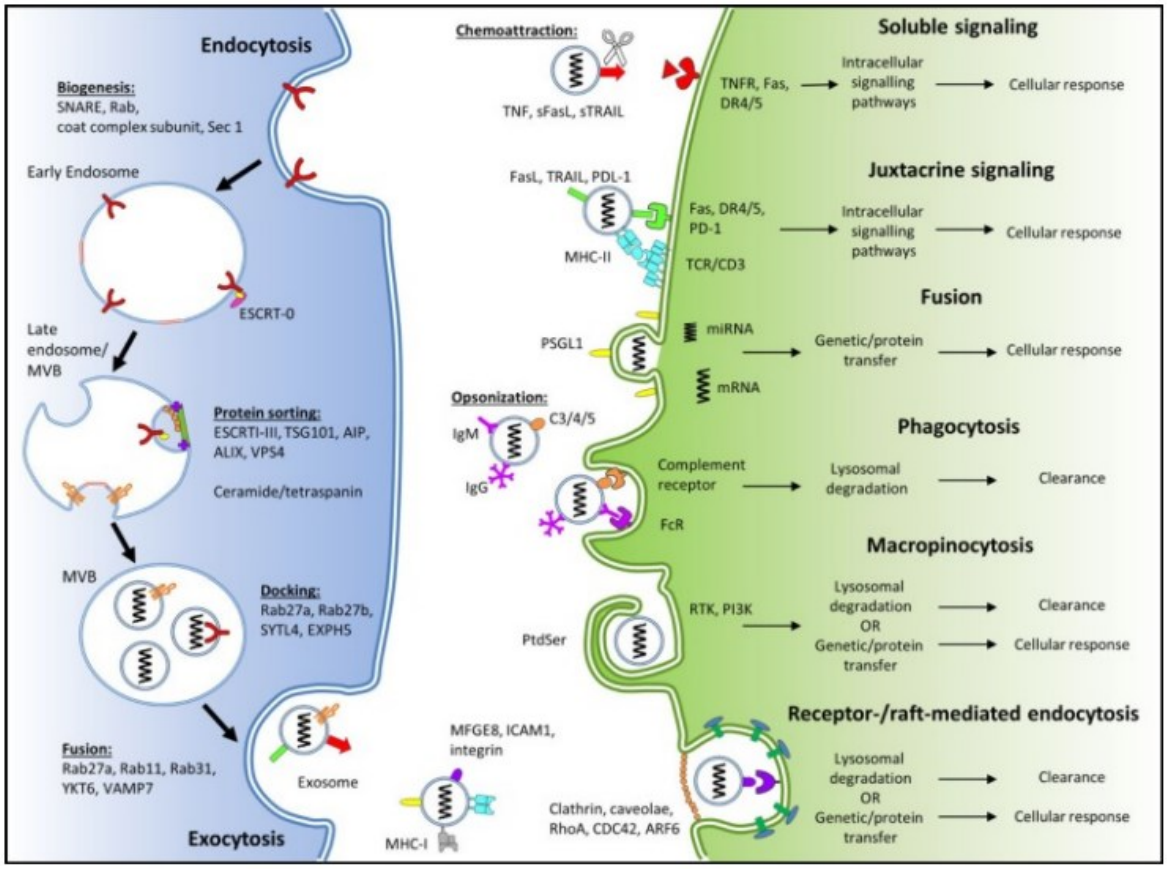

Figure 1.2. Biogenesis and internalization of exosomes [59]

1.8.2. Uptake of Exosomes into the Target Cell

Exosomes can be absorbed into the cell by the target cell in three different ways after they are released into the environment. Proteases in the target cell's plasma membrane interact with exosome membrane proteins via receptor contact. Soluble ligands are released at the places where target receptors on the cell surface interact. Exosome payload is transported into the lumen via selectively permeable intracellular signals. Although no particular receptors drive exosome absorption into the cell, a vast variety of proteins with possible receptor qualities, such as ICAM-1 for APCs and Tim1/4 for B cells, are present on their surface. Heparan sulfate proteoglycans (HSPGs) serve as effective receptors for exosomes produced from cancer cells. It has been demonstrated that enzymatic corruption of HSPG on the cell surface dramatically inhibits exosome absorption into the cell [56].

Exosomes move cargo by attaching to the cell membrane of the target cell through cytoskeletal proteins, metabolic enzymes, and membrane lipids [62].

When the membrane of the target cell and the membrane of the exosome fuse directly, the payload in the exosome is taken into the cytoplasm. The CD9 tetraspanins 5 on the surface of dendritic exosomes allow them to fuse directly with the target cell membrane and deliver their payload to the cytoplasm of the target cell [63].

The actin-cytoskeleton and phosphatidylinositol-3-kinase-dependent phagocytosis convey the exosome inside the cell as a whole during phagocytosis (internalization). The payload transported by the exosome is dispersed into the cytoplasm via processes within the cell [64].

1.8.3. Functional Applicaitions of Exosomes

At first, it was assumed that exosomes were the tools responsible for removing unnecessary cell molecules, but as a result of the researches, it was revealed that they have an important place in maintaining the body balance by taking part in intercellular communication and transport as well as these functions. Given the fundamental roles of exosomes in the control of biological processes, it has been recognized that these extracellular vesicles play a vital part in disease pathogenesis and have been discovered to have a role in many bacterial, parasite, or viral infectious illnesses. In addition to these functions, they play critical roles in immunological responses and immune homeostasis. Furthermore, exosomes are known to be engaged in immune system communication, facilitating regulation with both immunostimulatory and suppressive effects [65].

The components of exosomes, which vary depending on the condition of the cells from which they arise, allow these nanovesicles to be employed in illness detection and therapy. Exosomes derived from diseased cells have particular cancer cell proteins, according to research on the diagnostic use of exosomes in cancer. For example, it has been established that prostate cancer indicators detected in plasma and serum exosomes retrieved from the body are dictated by the findings of investigations on prostate cancer cell lines. Especially with the discovery that exosomes are natural carriers of RNA, it was hypothesized that they could be used as vehicles for the transport of exogenous therapeutic cargoes. It is thought that miRNAs with various oncogenic properties, which are carried by exosomes other than protein, have an important place in the diagnosis of cancer disease [66].

Exosomes produced by endothelium cells, smooth muscle cells, and cardiomyocyte cells that supply blood circulation in the heart muscle have been found to play an indispensable role in the evolvement and progression of coronary heart disease in studies other than cancer.

Exosomes produced by these cells into the environment have been shown to carry crucial biological information for the detection of coronary heart disease [67].

The high biodistribution and stable structures of exosomes in plasma facilitate their use in therapeutic applications, and their use on their own or as a carrier for an exogenic molecule is one of the leading therapeutic applications [67].

Studies have shown that exosomes also create an immune response against tumors. Immunotherapy, which helps to destroy cancer cells by supporting body resistance, is an area that attracts attention especially in cancer treatment [68]. Due to their immunomodulatory capabilities, the concept that exosomes can be utilized in cancer immunotherapy is gaining ground. In the early phases of the cancer/immune cycle, dendritic cells have been discovered to destroy cancer cells by activating T cells. Exosomes generated from dendritic cells have been found to have the ability to promote immune cell-dependent tumor rejection by activating T cells. In light of these findings, the use of exosomes as a novel therapeutic method for disorders such as cancer appears promising. Exosomes are also known to provide a protective function in the neurological system. Exosomes are implicated in neural protection, nerve regeneration, and neuronal growth, according to several studies [69].

1.8.4. Plant Exosomes

PENs are being researched as an alternative to MDEs for circumventing the technical restrictions of mammalian vesicles. PENs have considerable potential for use in disease treatment and the development of nanocarrier DDS with variable dose delivery [70]. Wang et al. created multifunctional PENs as nanovectors for brain tumor medication delivery. Due to nanovector stability, the PENs aggregated in specific tissues *in vivo* and circulated for an extended period of time in the periphery [71].

As molecular cargo is transferred to the plasma membrane for budding and release, numerous crucial stages in PEN biogenesis differ from MDEs, and they may have distinct features and activities depending on their objectives. MVB-mediated exosome-like nanovesicle secretion in plants needs conclusive verification [72].

Novel pharmaceutical development methods must be used more often for more effective ailment therapy. Therapies based on PENs have the potential to be an effective new means

of treating cancers, inflammatory conditions, and immune-system problems. Because of the naturally occurring biochemicals, food plants including grape [71], grapefruit [72], ginger, carrot, and apple [73], among others, produce PENs, which are capable of producing therapeutic effects. The effects of PENs might be both physiological and pathological. Targeting and controlling the microbes that live in the gut allows ginger-derived exosomes, also known as GDENs, to inhibit the growth of tumor cells and improve symptoms of inflammatory bowel disease (IBD). PENs are thought to be safer than manufactured nanocarriers such as metal-based nanoparticles (gold/silver), carbon-based nanoparticles, and nanoparticles based on copolymers, such as gold/silver nanoparticles [73].

1.8.5. Exosome Isolation Methods

Studies carried out to understand the biological properties of exosomes since their discovery have revealed that these nanoscale structures have very important physiological properties. Therefore, the isolation of exosomes is an important start for studies planned in both basic medicine and regenerative medicine. Isolation techniques consist of general techniques such as centrifugation, filtration, immunological, chromatography and polymer-based precipitation [74].

1.8.5.1. Centrifugation

Ultracentrifugation is the most thoroughly used and accepted gold standard isolation method for exosome isolation. This method is an optimized technique with a high rotational speed of 100,000 g. There are two types of techniques, differential and density gradient, for exosome isolation by ultracentrifugation method [75].

Johne-Stone et al. first developed the differential ultracentrifugation method and later this technique was optimized by Thery. The cell culture supernatant passes through successive centrifugation steps at different speeds and times. With this method, particles of different weights and sizes in the sample are separated after each centrifugation step. The pellet obtained in the last centrifugation step consists of exosomes [74].

In the density gradient technique, samples are subjected to ultracentrifugation in a dense medium such as sucrose. In this technique, the sample is separated into its fractions depending on the mass, size and density of the particles in it. At the end of this method, while the exosome pellets are collected in the region of density between 1.10-1.18 g/mL, protein

aggregates with higher density accumulate at the bottom of the tube. While the advantage of this technique is to obtain exosomes of high purity, the disadvantage is that it works in low volumes and the centrifugation time is long [76].

1.8.5.2. Ultrafiltration(UF)

This method is based on the same basic filtration methods based on particle size and molecular weight, that is, exosomes are isolated with membrane filters suitable for their sizes [76].

Although it is a faster isolation method compared to the ultracentrifuge method, it does not require special equipment. The disadvantages of ultrafiltration are that if the force applied during the method breaks up large molecules, the isolation result is adversely affected. In addition, exosomes can adhere to membrane filters and clog these filters, resulting in loss of exosomes [76].

1.8.5.3. Size Separation Chromotography

This chromatography method is used as a method for separating the components of the solutions according to their size. In this chromatography method, exosomes are separated from other bionanomolecules without damaging their functions and morphology. The advantages of this method are that it is a simple and easy method as well as a short isolation time. The disadvantages are that the exosome yield is low and aseptic conditions are required against the risk of contamination of the colon used [77].

1.8.5.4. Immunological Isolation

Many techniques have been developed for immunological isolation. Advantages: While high purity exosomes can be obtained, exosome-specific groups can be isolated. The disadvantages are that it is not suitable for high volumes, it is an expensive method, and antibodies specific for exosome ligands are needed [78].

1.8.5.5. Polymer Based Precipitation

In this method, the sample to be isolated from the exosome is mixed with a polymer-based precipitation solution. In order for the precipitation reaction to occur, polymers such as polyethylene glycol (PEG) or salt solutions such as sodium acetate are used. The isolation process continues with incubation and low speed centrifugation steps. After the

centrifugation steps are over, the part where the exosome is located is collapsed to the bottom of the tube (Figure 1.3.). The advantage is that it is an easy and short-term method. The disadvantages are the low purity in obtaining exosomes, the precipitation reaction in non-exosome structures and the purification steps applied to remove the polymer [79].

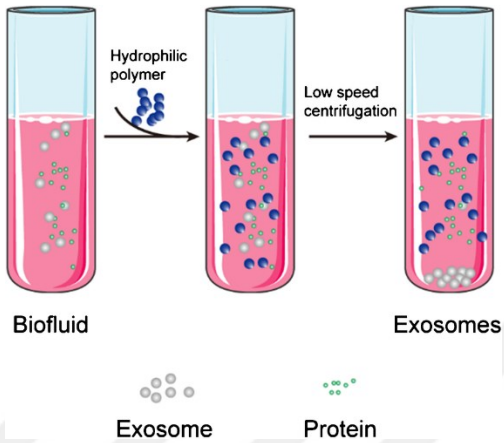

Figure 1.3. Exosome separation based on polymer precipitation [80]

1.9. AIM OF THE STUDY

Observe the effects of the exosomes, obtained from wheat grass and peppermint plant juice, on adipogenic differentiated human adipocyte-derived stem cells (hADSCs) utilizing real-time polymerase chain reaction, MTS cytotoxicity test, oil-red staining, cellular uptake of exosome, immunocyto chemistry and enzyme-linked immunosorbent assay.

2. MATERIALS

2.1. CHEMICALS

The Chemicals which were used during this thesis was listed below:

• Dulbecco's Modified Eagle's Medium, high glucose, Gibco 41966, Thermo F. Scientific

• Dulbecco's Modified Eagle's Medium, low glucose, Gibco 31885, Thermo F. Scientific

• Fetal Bovine Serum (FBS) cell culture tested, Gibco 10500-056, Thermo F. Scientific

• Penicillin-Streptomycin, Gibco 15140-122, Thermo F. Scientific

• Dimethyl Sulphoxide (DMSO), Santa C. Biotechnology sc-20258

• Phosphate Buffered Saline (PBS), Lonza BE17-517Q

• Trypsin-EDTA Gibco 25200056, Thermo F. Scientific

• Polyethylene glycol, Sigma81310, USA

• Dextran, Sigma 9004-54-0, USA

• TRIzolTM Reagent, 15596-018, ThermoFisher Scientific

• Bovine Serum Albumin (BSA) $0332-100G, LifeScience

• Mounting media F4680-25, USA

2.2. INSTRUMENTS

The instruments which were used during this thesis was listed below:

• Laminar Flow Cabinet, Heal Force Class II Biosafety Cabinet A2

• CO2 incubator, InVitro Cell ES NU-5800, NuAire, USA

• Inverted Microscope, Nikon, TS100,Netherlands

• -80^{o}C freezer, New Brunswick, Germany

• Elisa Microplate reader, Bio-techELx800, USA

• Centrifuge, Sigma A. 3-18KS, Germany

• Nanodrop, Thermo F. Scientific, USA

• Thermal Cycler, Biorad Mycycler 1709703, USA

• CFX96 Real-Time PCR, Bio-Rad, C1000 Touch, USA

• Vortex, Dragon Lab, MX-F, China

• Incubate-shaker, New Brunswick Innova, USA

• Magnetic Stirrer, Heidolph MR, Germany

• Fluorescence Microscope, Axio Vert. Zeiss, Germany

• Scannning Electron Microscope, LSM 700, Germany

2.3. EQUIPMENT

The lab equipments which were used during this thesis was listed below:

• Cell culture flasks, T-25, T-75, T-150, Switzerland

• Serological Pipettes 5,10,25,50 mL CAPP, Germany

• Micropipette, 2231300002, Germany

• Micropipette tips, 301-03-051, Axygen, USA

• Microcentrifuge-tubes 1.5, 2 mL, 078.03.002, 078.03.003, Isolab, Germany

• Falcon tubes 15 and 50 mL, Isolab, Germany

• Cryovials, 091.11.102, Isolab, Germany

• Graduated Cylinder, Isolab, Germany

• Filter 0.22 µm and 0.45 µm TPP, Switzerland

• Hemacytometer, Sigma Aldrich, Z359629, USA

• Coverslip, Sigma, Z375357, USA

2.4. KITS AND ASSAYS

• StemPro Adipogenesis Differentiation Kit (Gibco, A1007101)

• RayBio. Human PPAR-gamma ELISA Kit

• The Human LPL ELISA kit, BMS2014-3

• QuantiTect SYBR Green PCR Kit(204145) Qiagen

• iScriptTM cDNA synthesis kit, (cat#1708890), BioRad

• Lowry Protein Assay Kit from BioRad

2.5. ANTIBODIES

• PPAR-gamma antibody, Abcam, USA

• LPL antibody, Abcam, USA

• Goat anti-rabbit and mouse, (Alexa Fluor. 647), Abcam, USA

• DAPI (DS1306), ThermoFischer Scientific, USA

2.6. CELL LINE

• hADSCs, human adiposed stem cells, ATCC

3. METHODS

3.1. EXOSOME ISOLATION OF *T. AESTIVUM LIN.* AND *MENTHA PIPERITA*

T. aestivum Lin.(wheat grass) and *Mentha piperita* juices are used to isolate exosomes. *T. aestivum* seeds were obtained from Ceyhan 99 in Adana. Before wheat seeds grown under light conditions, they were sown in moistened perlite and cultivated at roo temperature for 36 to 48 hours under dark conditions. When the length of the wheat grass leaves reached roughly 10 cm (around 5-7 days after the grass was grown), it was harvested, rinsed, and pressed in distilled water using a laboratory mixer. Following the washing process, the wheat grass juice was centrifuged at 1000 rpmi for 10 minutes. Exosome isolation was carried out in line with the aqueous two-phase system (ATPS) approach defined in the literature [87].

Dextran (DEX), Poly-ethylene glycol (PEG) were combined in a 1:1 ratio with distilled water to produce an ATPS isolation solution. So that, 20 mL of ATPS solution and 20 mL of plant lysate were combined and centrifuged at 1000 xg for 10 minutes to allocate an aqueous two phase solution. After the centrifugation stage, 80% of the ATPS-plant lysate solution (volume) was thrown out, and the identical amount of ATPS isolation solution was added and centrifuged at 1000 xg for 10 minutes as a washing step. In order to enhance the purity of exosomes, the process of washing is repeated twice. After the washing processes, the top phase was thrown out and the exosome-containing underside phase was filtered through 0.45 m and 0.22 m filters.

3.2. EXOSOME CHARACTERIZATION AND QUANTIFICATION

Exosomes which were isolated from wheat grass and peppermint juices were subsequently analyzed using a nanoparticle tracking analysis (NanoSight). After these process, the Lowry Protein Assay was used in order to determine the exosome concentration.

The NTA technique with a 488 nm laser was used to compute exosome quantification and size distribution nm. According to the manufacturer's instructions, wheat and peppermint exosomes were diluted with distilled water in the 1:40 concentration range. The video collected nanoparticles at 60 seconds intervals for at least five captures, and the test was performed using the NTA software version 3.3.301.

The Lowry protein assay was used to determine the quantity of exosomes. 4 µl of exosomes were blended with 16 µl of distilled water, and 5 µl of the mixture was transferred into three wells of 96-wellplate. In each well, 25 µl of reagent A solution and 220 µl of reagent B solution were added into each sample and incubated for 30 minutes in the dark conditions at room temperature. After 30 minutes incubation, the absorbance of the samples was measured at 490 nm using a Multiscan Spectrophotometer.

3.3. CELL CULTURE

Human adipose-derived stem cell (hADSCs) was cultured in DMEM Low media, both supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin (1% PS) and 10% (v/v) FBS (Fetal Bovine Serum). This cell line were cultured at 37ºC, 5% CO_2 (v/v and 95% air (v/v).

3.4. CELL PASSAGING

Cell passage was necessary to execute when cells reached 70-80% confluency. The cell monolayer was washed with 1X Phosphate-Buffered Saline(pH 7.4). After washing, cells were removed from the cell culture flask using 0.025% (w/v) trypsin for 5 minutes at 37ºC. After the cells had been trypsinized, 10% (v/v)fetal bovine serum (FBS)-containing medium was poured into the cell suspension to neutralize trypsin activity before collecting the cells. After that, the cell suspension was centrifuged for five minutes at a speed of 300 x g, the supernatant was discarded, and the cell pellet was put in new growth medium.

3.5. CELL COUNTING

Following the procedure for cell passaging that was outlined earlier, 10 microliters of the cell suspension were transferred into the hemocytometer. A 4x objective was used in order to count the cells in the central square of the hemocytometer while the microscope was in its inverted position.

The following calculation was used to get the total cell number per mililiter:

Cell number/ml= ‘counted cell number x dilution factor, factor/ mm2 x chamber depth’.

3.6. CELL CRYOPRESERVATION

The freezing mix solution was made by adding 10% (v/v) dimethyl sulfoxide (DMSO) and 90% (v/v) frozen bovine serum. Following the phase of centrifugation described in section 3.2.4, the cell pellet was resuspended in 1 milliliter of freezing mixture and placed in a cryovial that had been labeled. For long-term preservation, cryovials were first kept at a temperature of -80ºC, and then they were transferred to liquid nitrogen.

3.7. CELL THAWING

Cells were taken from liquid nitrogen and quickly warmed to 37ºC. To prevent cells from osmotic pressure increases, 5 mL of warmed growth medium was transferred drop by drop into the freezing mix solution which includes cells. After this step, the cell suspension was spun for 5 minutes at 1300 rpm. After the supernatant was thrown away, the cell pellet was put back together in full growth medium. The next day, the cell's media was replaced with new medium to eliminate all DMSO.

3.8. CELL VIABILITY

The MTS test was used to determine the viability of hADSCs. 5000 cells were put in each well of 96-well plates and left there for 24 hours in humidified conditions at 37°C with 5% CO_2. The next day, wheat grass and peppermint exosomes were given to cells at varied doses: 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL. The cellular viability was then determined using the MTS test, as directed by the manufacturer. The proportion of cell viability was evaluated by setting the absorbance value of untreated cells to 100%.

3.9. ADIPOGENIC DIFFERENTIATION

Human adipose stem cells were planted in 6-well and 96-well plates and cultivated in DMEM low glucose until confluency reached 80-85%. After this point, the medium was replaced with growth media (DMEM low glucose, 10% FBS, gentamycin reagent) for 2 hours. Simultaneously, an adipocyte differentiation basal medium containing 11 µl gentamycin and 10 ml of adipogenesis supplement was prepared. After 2 hours of growth media incubation, adipogenic differentiation was initiated using Thermo Fisher's StemPro Adipogenesis Differentiation Kit. Every two days, the differentiation media was replaced with new medium. Adipogenic differentiation was accomplished after 11 days.

3.10. OIL-RED O STAINING

Oil-red staining was performed on the cells following exosome treatment. 0.035 gr oil red was combined with 10 ml isopropanol and vortexed well before waiting 10 minutes at room temperature. At the same time, 6 mL of distilled water and 4 mL of isopropanol were combined to make Solution 1. Following the incubation of the oilred-isopropanol combination, solution 2 was produced using 4 mL of the oilred-isopropanol mixture and 6 mL of distilled water. To stain the cells in a 96-well plate, 9600 l of 2% (v/v) PFA was used for 20 minutes at room temperature. Following fixation, solution 1 was applied to a 96-well plate and incubated for 5 minutes at room temperature. After discarding solution 1, 100 l of solution 2 was poured to each well of a 96-well plate. The incubation time was 10 minutes at room temperature. Following that, each well was scrubbed 4-6 times with distilled water. Following the washing process, 100 microliter of PBS solution was transferred to each well. At 40X, stained cells were viewed and photographed using a light microscope.

3.11. RNA ISOLATION

Human adipose derived stem cells were seeded at a density of $1.5x10^5$ cell/well in 6-well plate. During adipogenic differentiation, the cells were treated with 100 µg/mL wheat and peppermint exosomes the next day. After 11 days of treatment with exosomes, conditioned media was collected and each well rinsed once with 1X PBS (pH7.4, 37 °C). The Trizol solution was added to each well in accordance with the manufacturer's instructions. The cell suspension in trizol was transferred to a fresh 1.5mL tube, and chloroform was added to each cell sample. Each sample was vortexed many times and samples were incubated for 2-3 minutes at 37 °C. After incubation, each sample were centrifuged at 12000 x g for 15 minutes at 4 °C. Following the centrifugation step, three different phases were seen. The upper phase is the one in which the RNA is present. The second phase has the same yellowish hue as the protein phase, while DNA was found in the third phase. Then, the RNA phases of samples were transferred to a new 1.5mL eppendorf tubes. For cleaning of the samples, pre-cooled isopropanol and the quantity collected from the first phase were mixed, and the eppendorf tubes were vortexed at few minutes. After incubating the samples for 10 minutes at room temperature, samples were centrifuged for 10 minutes at 12000 x g at 4 °C. The supernatants of samples after centrifugation wert removed, and each sample was mixed with 75% ethanol in pre-cooled form. The samples were centrifuged at 7500 rpm for 10 minutes at 4 °C. After centrifugation process, the ethanol was removed, and the pellets were air dried. Following

that, each sample was diluted with 50 microliter of RNase/DNase free water, and the RNA concentration was determined using a nano drop.

3.12. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

BioRad iScript™ cDNA Synthesis kit was use for the translation of RNA to complementary DNA (cDNA). According to manufacturer's protocols(Table 3.1.), master mix was prepared. After reaction accordance to Table 3.2. , cDNA concentration of samples were measured with using NanoDrop.

Table 3.1. cDNA reaction mix preparation(BioRad)

Components	**Volume per Reaction (µl)**
5X iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	Variable
RNA Template(100fg-1 µg total RNA)	Variable
Total Volume	20

Table 3.2. cDNA synthesis reaction protocol(BioRad)

Priming	5 minutes at 25ºC
Reverse Transcription	20 minutes at 46ºC
RT inactivation	1 minute at 95ºC
Hold	4ºC

3.13. QUANTITATIVE POLYMERASE CHAIN REACTION

In order to standardize the levels of gene expression, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used. This gene serves as a housekeeping gene. The QuantiTect SYBR Green PCR Kit, deionized water, and 10 pmol of each primer were mixed together in order to prepare the master mix for the RT-PCR. The master mix was then distributed to the respective wells for the RT-PCR. In order to complete the experiment with a volume of 10 ul, 800 ng of cDNAs were additionally added. The RT-PCR tests were carried out using a device called iCycler RT PCR (Table 3.3.). The gene expression data were standardized against their corresponding GAPDH values, and the results were shown as fold changes.

Table 3.3. RT-PCR reaction protocol

Cycle	Repeat	Step	Duration	Temperature
Initial Denaturation	1	1	5min	93ºC
Denaturation		1	60 secs	93ºC
Annealing		2	60 secs	55ºC
Extension		3	60 secs	72ºC
Final Extension	1	1	10 min	72ºC
Melt Curve	110	1	12 secs	-0.5ºC/cycle
Hold	1	1	-	4ºC

3.14. IMMUNOCYTOCHEMISTRY

Human adipose-derived stem cells were put into 8-well chambers at a density of 5x103 cells per well and left there overnight at 37oC in an environment with 5% CO2 and 95% (v/v) air to help the cells stick to the chambers. After overnight incubation, adipogenic differentiation was started for 11 days. During the adipogenic differentiation step, the control groups were treated with complete DMEM medium(low glucose) and the treatment groups were given 100 µg/ml of wheat and peppermint exosomes. The cells were incubated for 2 days. After 2 days incubation, treatment step was repeated to 11th day. Following the treatment step, exosomes and normal complete medium of the cells were discarded. The cells were fixed

with 4% (w/v) paraformaldehyde for 20 minutes at 4ºC (PFA). After that, permeabilization of the cells was done by adding 0.1% (v/v) Triton X-100 in PBS that had already been made. Triton X-100 was mixed with the cells for 10 minutes at room temperature (RT). Following the incubation, 1X PBS in 0.1% (w/v) Tween® 20 Detergent (PBS-T) was used to washed the cells three times for 5 minutes. Incubating cells in 5% (v/v) BSA (VWR Life Science) for 30 minutes at 4ºC with moderate shaking decreased non-specific antibody binding. Then, washing step with PBS-T was occurred three times for 5 minutes. After that, these cells were trated overnight with PPAR-gamma and LPL antibodies. The following day, cells were washed three times for 5 minutes in PBS-T to eliminate primary antibody residues. Secondary antibodies were goat anti-rabbit IgGH&L and anti-mouse IgGH&L (Alexa Fluor® 647). After washing step, cells were incubated with secondary antibody at 4ºC for 1 hour with modarete shaking. Cells were washed with PBS-T for 5 minutes three times to eliminate secondary antibody residues. Again in the dark at 4ºC, nucleus dye DAPI was added to the cells. After nuclear dye, cells were washed twice for 5 minutes with PBS-T. The slides were then sealed with mounting media and examined with a Confocal Laser Scanning Microscope (Zeiss LSM 700).

3.15. CELLULAR UPTAKE

Exosome uptake studies showed cell-taken exosomes. First, cell nuclei were stained with Hoechst dye at a final concentration of 1 µg/ml. Attached cells were coloured using CellTrackerTM Red CMPTX Dye Lyophilized CMPTX dye stock solution was produced in DMSO at 10 mM. Working solution is 25 M stock solution diluted in serum-free medium. The adhered cells were incubated at 37oC with 5% CO2 and 95% (v/v) air for 30 minutes. Exosomes are coloured using PKH26 Red Fluorescent Cell Linker Kit (#PKH26GL) following manufacturer's instructions. 1 ml of diluent c from the PKH kit is combined with 4 µl of the PKH26 dye and 1 ml of exosome (1:1 ratio) solution for 30 seconds. After 5 minutes at room temperature, exosomes and dye were analyzed. Once exosomes are colored, leftover dye is removed with centrifugation them at 750 xg for 2 minutes in exosome spin columns. Exosomes were delivered to red CMPTX-dyed cells in full media after leftover dye was eliminated. HADSCs were photographed at 1-, 3- and 24 hours.

4. RESULTS

4.1. EXOSOME SIZE AND PARTICLE NUMBER DETECTION VIA NTA

NTA, which is the first of the biophysical methods and an optical particle tracking method, can measure the concentration and size distribution of exosomes in the range of 1 nm to 2 µm. This method helps us to perform simultaneous analysis of the size, size distribution, concentration and phenotype of the particles. Important advantages of NTA are the detection of EVs with exosomes and the ability to measure nanoparticles with diameters as small as 30 nm. This method provides convenience and speed in sample preparation.

According to graph 4(a) , the average size of wheatgrass exosomes is 100.4 nm and the mode is 111.2 nm. In contrast, the exosome concentration was found to be $1.36x10^9$ +/- $8.33x10^7$ particles/ml, and the particle number per frame was 6.3+/-0.4 (Figure 4.1.).

The average size of pepprmint exosomes, as seen in graph 5(a), is 95.5 nm, whereas the mode is 102.9 nm. In contrast, the exosome concentration was determined to be 1.41 x 10^{11} +/- 2.11 x 10^{10} particles/ml, with 8.7 +/- 0.8 particles per frame (Figure 4.2.).

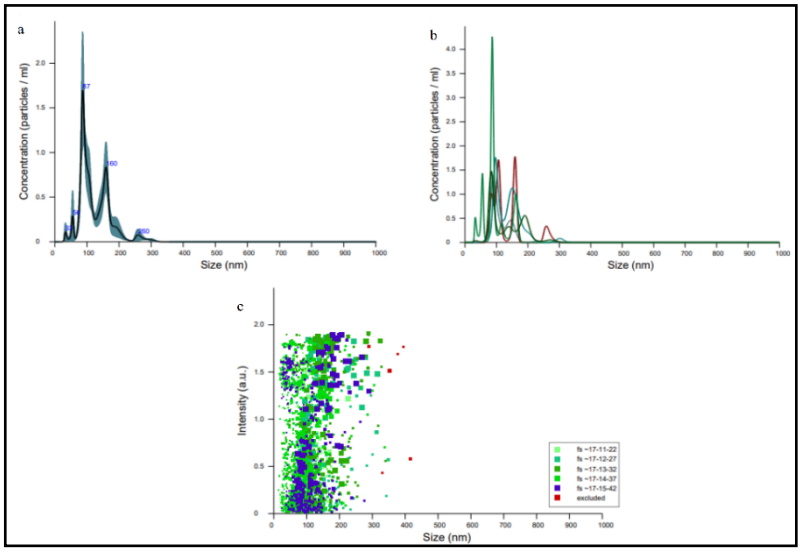

Figure 4.1. Nanoparticle tracking analysis results of wheat grass exosomes showing particle size, concentration and their variety. (a) Averaged Finite track length adjustment (FTLA) concentration / size, (b) FTLA concentration /size, (c) Intensity /size of exosome distribution. n =5 groups, Error bars indicate ±1 standard error of the mean

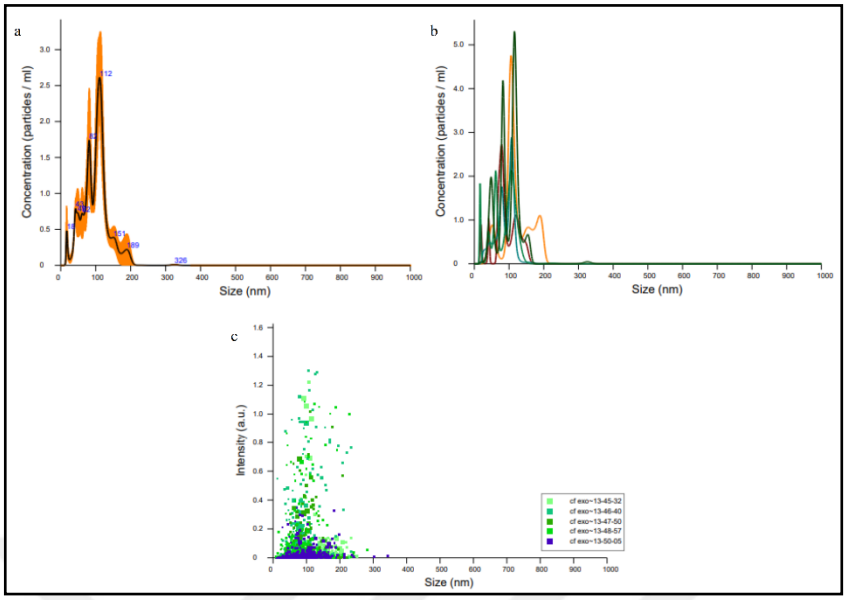

Figure 4.2. Nanoparticle tracking analysis results of peppermint exosomes showing particle size, concentration and their variety. (a) Averaged Finite track length adjustment (FTLA) concentration / size, (b) FTLA concentration /size, (c) Intensity /size of exosome distribution. n =5 groups, Error bars indicate ±1 standard error of the mean

4.2. TIME DEPENDENT CELLULAR UPTAKE OF EXOSOMES

The functioning of exosomes formed from wheatgrass and peppermint was evaluated using time-dependent cellular uptake to see if these exosomes may be taken up by adipose-derived stem cells as a sign of interkingdom communication. PKH26-dyed exosomes were administered to stem cells. Exosome internalization was seen at different time periods using fluorescence microscopy. Exosome internalization in adipose stem cells began around one hour after treatment, and the intensity of exosomes steadily increased over the next three, and twenty-four hours (Figure 4.3.).

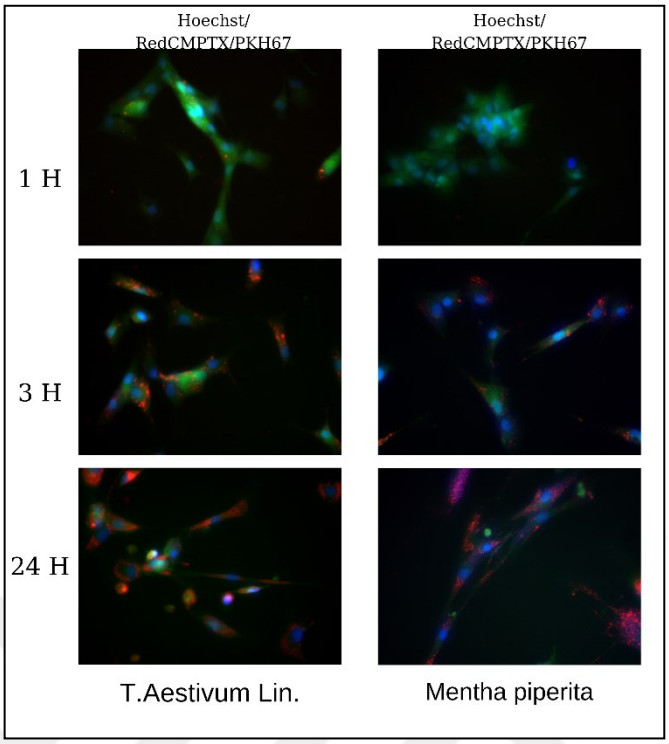

Figure 4.3. The absorption of exosomes by hADS cells is time dependent. (40X magnification, scale bar at 20µm)

4.3. CELL PROLIFERATION

The MTS test, which is dependent on the mitochondrial activity of the cells, was used to examine the impact that the exosomes had on the adipose derived stem cells after they had been characterized.

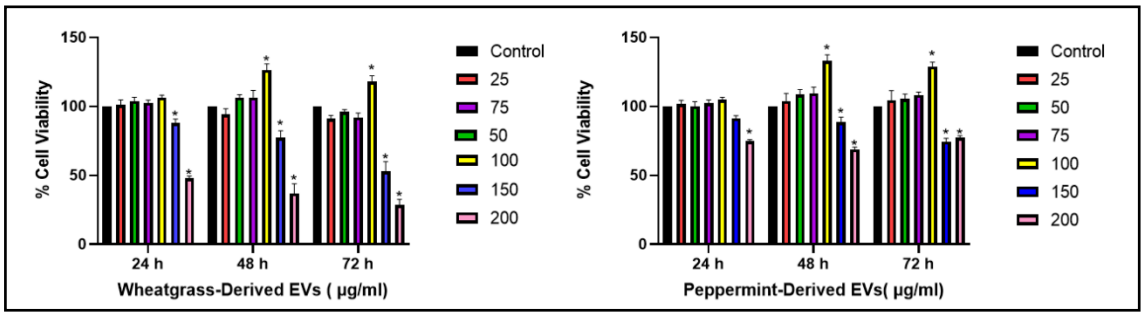

Figure 4.4. The effects of *T. aestivum Lin.*(a) and *Mentha piperita*(b) exosomes on adipose derived stem cell proliferation. *P<0.05, control was accepted 100% for each day respectively

According to Figure 4.4., 25-50-75-100-150-200 µg/ml doses of wheatgrass and peppermint exosomes were applied on adipose-derived stem cells, and no toxicity was observed at 25-50-75-100 µg/ml doses. A toxic effect was observed in cells treated with exosomes at 150 and 200 µg/ml concentrations. Differentiation experiments will be continued with the two highest non-toxic doses, and this dose will be reduced to one even as a result of oil red-o staining.

4.4. OIL-RED O STAINING

Oil Red O (Solvent Red 27, Sudan Red 5B, C.I. 26125, C26H24N4O) is a lysochrome (oil soluble dye), diazo dye used to stain neutral triglycerides and lipoproteins in the paraffin fraction and lipids in the frozen fractions. It is used to show fats and lipids in fresh tissue sections. Particularly, the display of fat cells and neutral fats is a common area of use. It is also used to detect where oils are found or drifted abnormally. Lipid vesicles were detected in the control group and the peppermint EVs-treated cell group. As shown in Figure 4.5., treatment of differentiated hADSCs with wheatgrass EVs markedly destroyed lipid vesicles. These results indicated that wheatgrass has potential antiobesity effects on hADSCs.

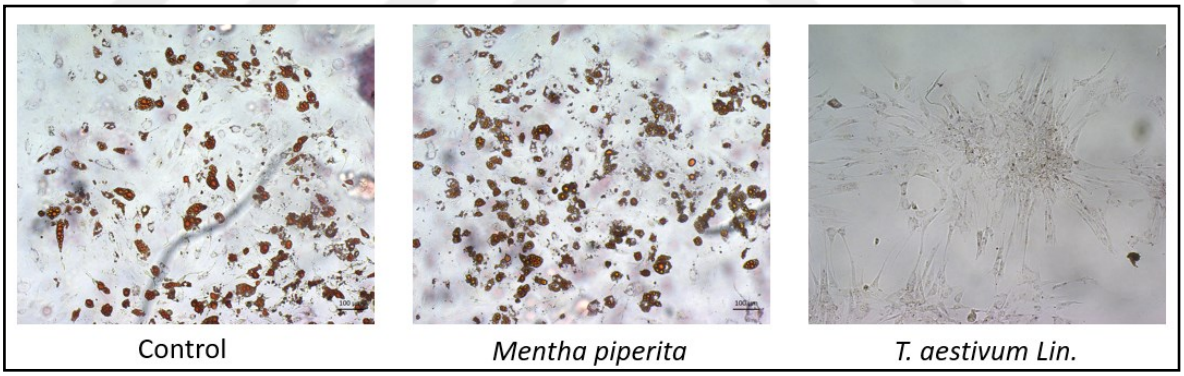

Figure 4.5. Effect of wheat grass and peppermint exosome on lipid formation on adipogenic differentiated cells

4.5. REAL TIME-PCR ANALYSIS

To comprehend the molecular mechanisms that *T. aestivum Lin.* and *Mentha piperita* exosomes affect, after cells undergoing adipogenic differentiation were examined with 100 µg/ml exosomes for 11 days, genes related to adipogenesis and obesity were evaluated by RealTime-PCR. Results designate that upon treatment with 100 µg/ml wheatgrass exosomes PPAR-gamma, adiponectin, LPL, Leptin and FTO levels decreased respectively however,

CEBP/alpha and alpha-p2 level did not changed significantly. Another result of treatment with 100 µg/ml peppermint exosomes, PPAR-gamma, adiponectin, CEBP/alpha levels increased by 4 folds. In addition, Alpha-p2, LPL, leptin and FTO mRNA expression levels increased by 3-, 5-, 5- and 6- folds upon peppermint exosome therapy (Figure 4.6.).

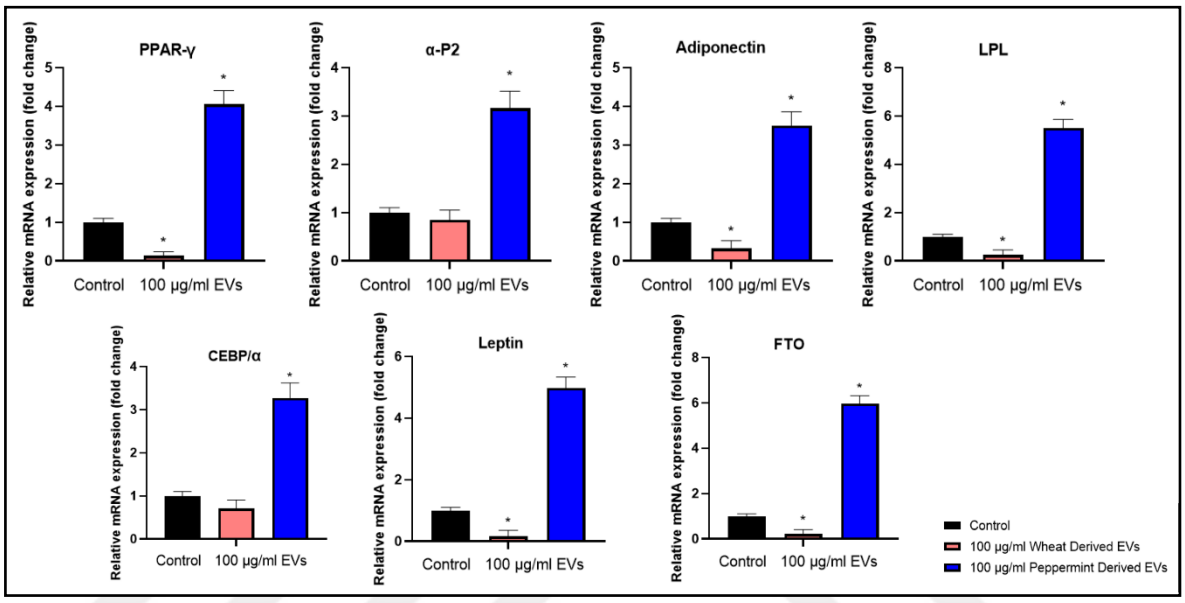

Figure 4.6. mRNA expression levels of adipogenic differentiated stem cells upon 100 µg/ml wheat and peppermint exosomes treatment. Abbreviations: Ppar-gamma: , Alpha p2:, Adiponectin: , LPL:FTO:, CEBP-alpha:, Leptin. Results were ecaluated by two-tailed multiple t tests. Data are shown with ± SEM, n=6, *P<0.05

4.6. ENZYME-LINKED IMMUNOSORBENT ASSAYS

4.6.1. PPAR-Gamma

Human PPAR-gamma ELISA was operated to evaluate PPAR-gamma levels on adipogenic differentiated stem cells upon wheat and peppermint exosomes application. The experiment revealed that treatment with *T.aestivum Lin.* resulted in a substantial reduction in PPAR-gamma expression levels, whereas treatment with *Mentha piperita* resulted in an increase (Figure 4.7.).

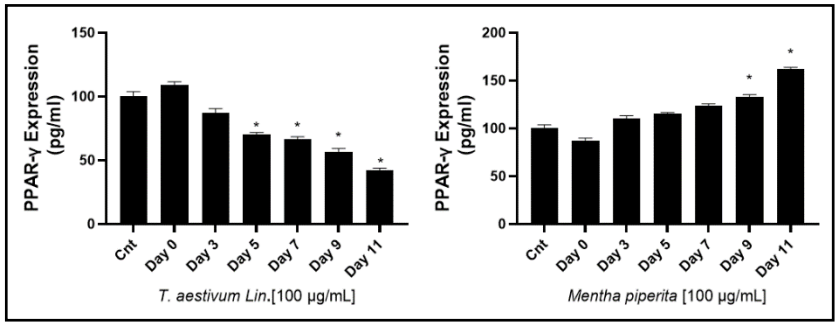

Figure 4.7. PPAR-gamma expression levels of adipogenic differentiated stem cells. Results were estimated by two-tailed multiple t tests. Data are shown with ± SEM, n=3, *P<0.01

4.6.2. LPL

Human LPL ELISA assay was performed to ascertain LPL levels on adipogenic differentiated stem cells upon wheat and peppermint exosomes application. According to the results come from the assay, LPL expression levels significantly downregulated in cells which was treated with *T.aestivum Lin.* and upregulated in cells which was treated with *Mentha piperita* (Figure 4.8.).

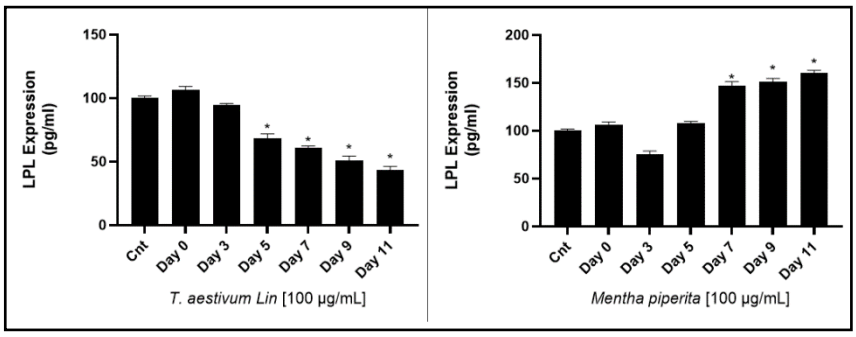

Figure 4.8. LPL expression levels of adipogenic differentiated stem cells. Results were investigated by two-tailed multiple t tests. Data are shown with ± SEM, n=3, *P<0.01

4.7. IMMUNOCYTOCHEMISTRY

Immunocytochemistry analysis was performed for seeinf protein localization and quantify protein expression levels on adipogenic differentiated cells. According to the Figure 4.9. and Figure 4.10., PPAR-gamma protein expression level cells which was treated with wheat grass exosomes significantly decreased while LPL expression level was significantly downregulated among HADSC cells after treatment with wheat exosomes. On the other

hand, Ppar-gamma expression level of adipogenic differentiated cells which was treated with peppermint exosomes was also impotantly increased while LPL expression level was significantly upregulated among HADSC cells after treatment with peppermint exosomes. In general, it was observed that the expression of genes related to adipogenesis and obesity was increased in the peppermint exosome-treated cells compared to the control group, and decreased in wheatgrass exosome-treated cells compared with the control group. Accordingly, it can be said that peppermint exosome induces obesity by upregulating these pathways.

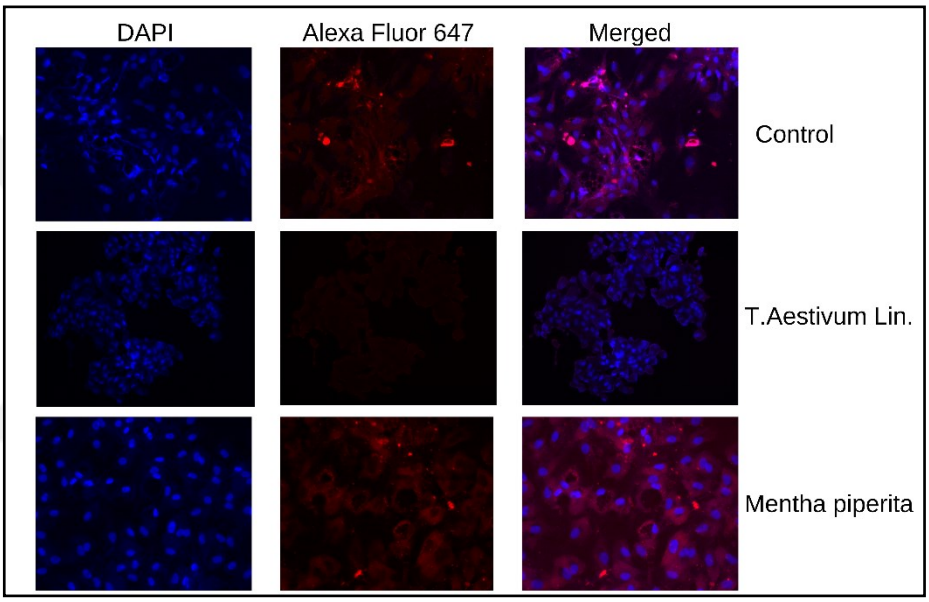

Figure 4.9. Localization and quantification of PPAR-gamma protein expression of adipogenic differentiated stem cells by confocal microscopy. Results were investigated by two-tailed multiple t tests. Data are shown with ± SD, n=3

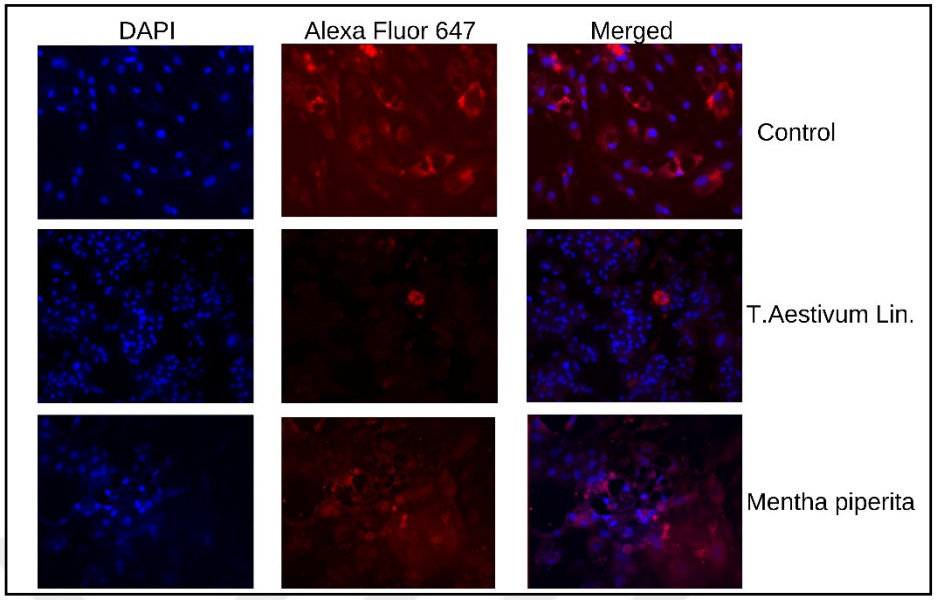

Figure 4.10. Localization and quantification of LPL protein expression of adipogenic differentiated stem cells by confocal microscopy. Results were investigated by two-tailed multiple t tests. Data are shown with ± SD, n=3

5. DISCUSSIONS

Today, obesity has started to become an important health problem in all countries and is established by the world health organization as excess fat accumulation in the body to the extent that it impairs health. According to the research conducted by the Turkish statistical institution, while the rate of obese individuals was 19.6% in 2016, an increase of 21.1% was observed in 2019. In terms of gender, in 2019, 24.8% of women were obese and 30.4% were pre-obese, while 17.3% of men were obese and 39.7% were pre-obese. It is aimed to develop a herbal treatment by investigating the mechanism of this disease, which is caused by the storage of weight as fat in the body due to many factors such as eating disorders, genetic factors, environmental and psychological effects.

Human adipose derived(HADSc) stem cells are used to create an *in vitro* system for experiments on fat metabolism. HADScs are cellularly exposed to adipogenic differentiation, creating a cellular fat deposition pattern. Thus, the fat tissue accumulated in humans is modeled *in vitro*.

Exosomes are nanosized secretory vesicles that serve a key function in cellular connection despite being considered of as the cells' rubbish bins. Extracellular vesicles (EV) are important for aging, cancer, infectious disorders, and more [69]. Increasing publications with low or insufficient extracellular content has prompted the challenge of providing a guide for all EV investigations to distinguish exosomes, microvesicles, and apoptotic materials. The size and biogenesis processes of these extracellular vesicles differ. MISEV(Minimal Information for Studies of Extracellular Vesicles – MISEV) recommends that exosomes be counted, and that the exosomes be observed using either electron microscopy or NTA[74]. In addition to this, the recommendation highly recommends that one observe the cellular uptake of the exosomes within the target cells. Exosomes were isolated from wheat and peppermint for the purpose of this thesis, and then those exosomes were characterized using the aforementioned criteria. Exosomes were quantified and their size distribution was determined by nanoparticle tracking analysis. According to the literature researches, the size distribution can range from 40 to 160 nm [70], and the mean exosome size of samples obtained by NTA is approprite within the exosome size limits. However, size disparities are

expected for the exosome composition since the exosomes originate from plants rather than biological fluids. Even though the particle count was determined by NTA, further research was guided by the protein content of the exosomes due to changes in size distribution. As a result, the Lowry protein assay was used to determine the exact number of exosomes present in the sample during the study.

In addition, cellular uptake experiment demonstrated the cross-kingdom connection between exosomes and stem cells as the exosomes became increasingly visible within the cells two hours after application. Even 24 hours after its administration, their presence within the cells is evident.

When examined in the literature, it is seen that exosomes obtained from wheatgrass juice and mint have not been used before for obesity. This is the first original study in this field using plant exosomes.

Wheat grass (*T. aestivum Lin*.) is mainly used as a concentrated source of nutrients, and its content includes vitamin A, vitamin E, vitamin C, calcium, magnesium, iron and amino acids. Wheatgrass contains antioxidant and anti-inflammatory chemicals, as well as anti-bacterial chemicals. It is seen that this plant increases hemoglobin production and is promoted as a complementary treatment in diseases such as diabetes and cancer. According to laboratory research, wheatgrass juice has antioxidant effects with vitamins A, C and E, so its health benefits are due to the vitamins, mineral fiber and antioxidant properties it contains [80].

The peppermint is a plant that forms the genus *Mentha* of the honeydew family, or usually as a peppermint leaf. Peppermint is very rich in vitamin A. It also contains plenty of calcium, magnesium, potassium, folate and omega-3 fats. Regular consumption of peppermint is known to be a great appetizer. The aroma of the plant facilitates digestion by helping to activate the salivary glands in the mouth as well as the glands that secrete digestive enzymes [81].

A crucial role in the processes that control fat deposition is played by adipogenesis, which regulates the growth of adipose tissue as well as the systemic energy balance that is frequently disrupted in obese individuals. Two of the most important transcription factors in the control of fat deposition are called PPAR-gamma and C/EBP-alpha respectively. Controlling the amount of fat that is deposited in MSCs through the up- or downregulation

of PPAR-gamma is what regulates adipogenesis. In addition, C/EBP-alpha is known to activate PPAR-gamma related pathways in order to cause adipogenesis [33].

It has been suggested that a viable strategy for the treatment of obesity and disorders connected to it would be to focus on inhibiting the activity of the transcription factors that are accountable for regulating adipogenesis.

The development of antiobesity drugs is difficult due to the fact that the development of obesity is effected by a number of different factors. These factors range from an imbalance in the metabolism of energy to mutations in the genes that are responsible for metabolism, and even differences in individuals' habits regarding the consumption of artificial foods.

In addition, antiobesity medications that have been produced up until this point have only shown a moderate degree of therapeutic efficacy due to the cytotoxic and other negative consequences that they have. As a result, innovative therapeutic techniques are required for the treatment of obesity. These approaches must be more effective, targeted, safe, and well-tolerated than current methods.

This current study aimed to investigate the activity of wheatgrass and peppermint derived exosomes with using *in vitro* approaches on adipogenic differentiation of human adipose stemcells and developing new generation obesity treatment strategies. First, six different concentrations of peppermint and wheatgrass derived exosomes to be used on cells ordered from ATCC, their potential cytotoxic effects on hADSCs were examined using MTS analysis on cell viability at 24, 48 and 72 hours. The doses of these exosomes at different concentrations dissolved in the medium were determined according to the Lowry protein assay, and no toxic effects were observed in the first four doses. To evaluate the effects on adipogenesis of the two selected non-cytotoxic doses for each exosome, hADSCs were stimulated to differentiate for 11 days. Following this initiation, Oil Red O staining, mRNA expression levels of aP2, LPL, adiponectin, CEBP-alpha, PPAR-gamma, FTO and Leptin, and protein levels of LPL and PPAR-gamma were confirmed in cells undergoing adipogenic differentiation. Adipogenic differentiation of the cells was achieved by applying differentiation medium to human-derived adipose stem cells for 11 days. During this process, the fat follicles were visibly seen under the microscope in adipogenically differentiated cells, exosome doses of 75 and 100 µg/ml were applied to these cells. This experiment continued for 11 days, with exosome treatment repeated every two days. At the

end of the 11th day, the experiment was terminated and the cells were stained with Oil Red O dye. Images were taken under the light-microscope and it was seen that the most effective dose was 100 µg/ml (Figure 4.5) and other experiments were continued with this determined single dose. Considering these results, it is seen that the most effective dose of exosome obtained from wheatgrass is 100 µg/ml. Further experiments were carried out, followed by the optimal dose of 100 µg/ml. According to the results obtained from the Oil Red O staining, it was observed that the exosome obtained from wheatgrass juice was suppressed in the treatment group, and even the loss of oil follicles was observed compared to the control group. In addition, in the adipogenic differentiation cell group treated with peppermint exosome, fat accumulation could not be suppressed and the fat follicles remained intact.

RT-PCR and ELISA analyzes were performed for treated cells after adipogenic differentiation to detect changes in both mRNA and protein levels, respectively. While the adipogenesis markers adiponectin, alpha-P2, LPL and Ppar-gamma were progressively expressed in the adipogenically differentiated control group and peppermint exosome-treated cell group, there were statistically significant decreases in these markers in hADSC cultures treated with wheatgrass juice. At the same time, while the expression of obesity markers Leptin and FTO increased in the control group and the cell culture group treated with peppermint exosome, significant decreases were observed in the cell culture group treated with wheatgrass. When the results were examined, it was observed that the genes associated with adipogenesis and obesity were upregulated in the mint exosome-treated cells, and as a result, it can be concluded that the mint exosome induces obesity. In addition, when the genes related to adipgenesis and obesity were examined in the results obtained from cells treated with wheatgrass exosome, these genes were downregulated and the destruction of fat follicles in the cellular was observed.

These results are pioneering in opening a whole new discussion about the use of exosome-based compounds derived from wheatgrass juice in the treatment of obesity and comorbidities. In the present study, it was observed that wheatgrass juice had a suppressive activity after the preadipocytes, the predominant cell type in adipose tissue, differentiated into mature adipocytes, and mature adipocytes were observed to shrink.

Medicines that target the transitions between the three basic mammalian adipocyte types (white(WAT), brown(BAT) and beige adipose tissue) are of interest in the fight against

obesity. To what extent wheatgrass exosomes influence the differentiation of WAT adipocytes into BAT adipocytes, which are thermogenic and heat-dissipative, is an interesting question. The consistent rise in adiposity and insulin resistance in obese mouse model may be attributable to a chronic inflammatory response that enhances adipolysis and increases plasma levels of free fatty acid. This response is liasised by an intense and unusual accumulation of macrophages in obese WAT. According to some estimates, macrophages account for only around 5% of the cells in fatless adipose tissue but as much as 50% of the cells in obese adipose tissue. For a thorough assessments of exosome-based compounds as obesity-fighting factors, it is important to determine the effect of wheatgrass exosome on the switch from antiinflammatory to proinflammatory function of macrophages in fatless versus obese-adipose tissue, as well as on macrophage metabolic activity.

6. CONCLUSIONS

In conclusion, this is the first study that proves the effects of wheat grass and peppermint exosomes on adipogenic differentiated stem cells from hADSCs. NTA was utilized to characterize *Triticum aestivum Lin.* and *Mentha piperita* exosomes. Following adipogenic differentiation of hADSCs, various dosages of wheat grass and peppermint were administered to cells using the MTS cytotoxicity test and non-toxic two doses were chosen. Oil-Red staining and real-time polymerase chain reaction were used to investigate the impact of these exosomes on cells. Real-time PCR was used to evaluate the expression levels of adiponectin, LPL, PPAR-gamma, Leptin, CEBP-alpha, FTO and aP2. In addition, during adipogenic differentiation of hADSCs, the effect of the peppermint exosome on these cells was examined, it was observed that the genes were upregulated and induced obesity. When the effect of wheatgrass exosome on adipogenic differentiated cells was examined, it was observed that it down-regulated genes associated with adipogenesis and obesity, and fat follicles were not seen in the cells in Oil-red O staining. Based on this observation, the therapeutic potential of wheatgrass exosome may perform a role in the development of biotechnological drugs that can be used in the treatment of obesity and obesity-related diseases. At the same time, it is seen that there may be the possibility of using peppermint exosome in the livestock sector, in the feed industry and in the treatment of patients who cannot gain weight to increase meat yield. Wheatgrass and peppermint exosomes may have a role in the fight against obesity, but further study is needed to understand its effect on the metabolic activities and plasticity of all the key cell types in the adipose niche. This is an initial study and it will be possible to develop new drugs after complementary preclinical studies and subsequent clinical studies are completed.

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APPENDIX A: ENZYME-LINKED IMMUNOSORBENT ASSAYS

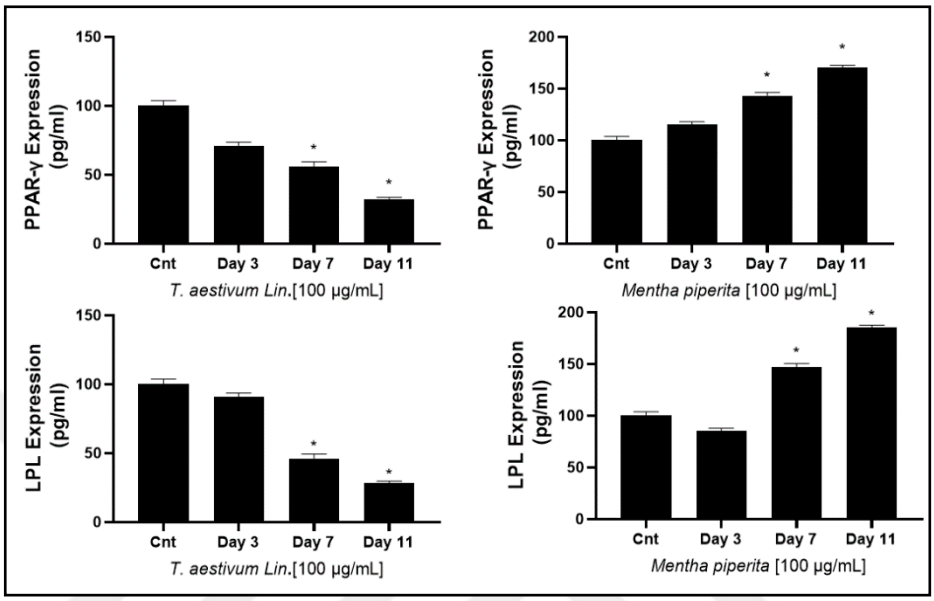

Figure A.1. PPAR-gamma and LPL expression levels of adipogenic differentiated stem cells. Control was treated with growth medium. Results were analysed by two-tailed multiple t tests. Data are shown with ± SEM, n=3, *P<0.01

APPENDIX B: LIPIDOMIC PROFILE OF *T.AESTIVUM LIN.* AND *MENTHA PIPERITA*

Table B.1. List of fatty acids identified in *T. aestivum Lin.*

Common Name	IUPAC Name	%
Carbazole	4-Methyl-9H-carbazole	36.68
Benzamide	N-(2,2-Dichloro-1-hydroxyethyl)-4-fluorobenzamide	63.32

Table B.2. List of fatty acids identified in *Mentha piperita.*

Common Name	IUPAC Name	%
Ammonium glutamate	Ammonium 5-oxido-5-oxonorvaline	15.58
Flufenisal	4-Acetoxy-4'-fluoro-3-biphenylcarboxylic acid	55.15
Propylparaben sodium	Sodium 4-(propoxycarbonyl)phenolate	29.27