



**REPUBLIC OF TURKEY
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY
UNIVERSITY**

**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOENGINEERING**

**INVESTIGATION OF THE EFFECT OF *B. sperguliofolius* EXTRACTS
ON INSULIN-RESISTANT 3T3-L1 ADIPOCYTES**

**Gizem Ece DERİCİ
MASTER OF SCIENCE**



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ADANA 2021

I hereby declare that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all information that is not original to this work.

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ABSTRACT

INVESTIGATION OF THE EFFECT OF *B. spergulifolius* EXTRACTS ON INSULIN-RESISTANT 3T3-L1 ADIPOCYTES

Gizem Ece DERİCİ

Department of Bioengineering

Supervisor: Asst. Prof. Dr. Sibel ÖZDAĞ February

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Diabetes mellitus (DM) is a metabolic disease with chronic hyperglycemia characterized by metabolic outcomes due to insufficient insulin secretion and/or insulin effect defect. DM often causes cardiovascular diseases and various complications on the eye, kidney and nervous system. Type 2 DM (T2DM) accounts for approximately 90% of the total DM. Therefore, it is very important to investigate new therapeutic approaches for T2DM and alternative, natural agents that target molecules in potential signal pathways. Medicinal plants are very significant resources in the research of alternative new drug active ingredients. *Bolanthus spergulifolius* (*B. spergulifolius*) is one of the genera of the family Caryophyllaceae. In the present study, it was aimed to investigate the potential anti-diabetic effects in-vitro of *B. spergulifolius* extracts on 3T3-L1 adipocytes.

B. spergulifolius were extracted with methanol (MeOH), ethylacetate (EA) and Aqueous and evaluated for their total phenolic content (TPC) by Folin-Ciocalteu method. *B. spergulifolius* extracts showing highly TPC (Aqueous < MeOH < EA) and their different concentrations were carried out on preadipocytes differentiated in to mature 3T3-L1 adipocytes to investigate their half-maximal (50%) inhibitory concentration (IC₅₀) by using MTT assay. The IC₅₀ concentrations of MeOH, EA and Aqueous extracts were observed as $305.7 \pm 5.583 \mu\text{g/mL}$, $567.4 \pm 3.008 \mu\text{g/mL}$ and $418.3 \pm 4.390 \mu\text{g/mL}$ and were used for further experiments. A live/dead assay further confirmed the cytotoxic effect of MeOH, EA and Aqueous extracts (respectively, $69.75 \pm 1.70\%$, $61.75 \pm 1.70\%$, $70 \pm 4.24\%$ and for all $P < 0.05$). Also, effects of extracts on lipid accumulation in mature 3T3-L1 adipocytes were evaluated by Oil-Red O staining assay. The extracts effectively decreased lipid-accumulation compared to untreated adipocytes (for all $P < 0.05$). Moreover, effect of extracts on apoptosis regulated by the *Bax* and

Bcl-2 was investigated by qRT-PCR. The extracts significantly induced apoptosis by upregulating pro-apoptotic *Bax* expression but down-regulating anti-apoptotic *Bcl-2* gene expression compared to untreated adipocytes (for all $P < 0.05$). The *Glut-4* expression linked with insulin resistance was determined by qRT-PCR, Western-blot and Immunofluorescence staining. In parallel, expression of *Glut-4* in adipocytes treated with extracts was significantly lower compared to untreated adipocytes (for all $P < 0.05$). Extracts significantly suppressed cell migration after 30 h of wounding in a scratch-assay (for all $P < 0.05$). Cell morphology and diameter were further evaluated by phase-contrast microscopy, Scanning electron microscope (SEM), Immunofluorescence (with F-Actin) and Giemsa staining. The adipocytes treated with extracts partially lost spherical morphology and showed smaller cell-diameter compared to untreated adipocytes (for all $P < 0.05$).

In conclusion, these results suggest that extracts of *B. spargulifolius* causes to an induce apoptosis, a decrease lipid-accumulation, wound healing, *Glut-4* level and might contribute to the reducing of insulin-resistance in DM.

Key Words: *Bolanthus spargulifolius*, diabetes mellitus, insulin-resistant, 3T3-L1 adipocytes, Glucose transporter-4, anti-diabetic

ÖZET

***B. spargulifolius* EKSTRAKTLARININ İNSÜLİNE DAYANIKLI 3T3-L1 ADİPOİTLER ÜZERİNDEKİ ETKİSİNİN ARAŞTIRILMASI**

Gizem Ece DERİCİ

Biyomühendislik Anabilim Dalı

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Diabetes mellitus (DM), yetersiz insülin sekresyonu ve / veya insülin etkisi kusuru nedeniyle metabolik sonuçlar, kronik hiperglisemi ile karakterize metabolik bir hastalıktır. DM sıklıkla kardiyovasküler hastalıklara, göz, böbrek ve sinir sisteminde çeşitli komplikasyonlara neden olur. Tip 2 DM (T2DM), toplam DM'nin yaklaşık % 90'ını oluşturur. Bu nedenle, T2DM için

yeni terapötik yaklaşımların ve potansiyel sinyal yollarındaki molekülleri hedefleyen alternatif, doğal ajanların araştırılması çok önemlidir. Tıbbi bitkiler, alternatif yeni ilaç etken maddelerinin araştırılmasında çok önemli kaynaklardır. *Bolanthus spergulifolius* (*B. spergulifolius*), Caryophyllaceae ailesinin cinslerinden biridir. Bu çalışmada *B. spergulifolius* ekstraktlarının 3T3-L1 adipositler üzerindeki potansiyel anti-diyabetik etkilerinin in-vitro olarak araştırılması amaçlanmıştır.

B. spergulifolius, metanol (MeOH), etilasetat (EA) ve su ile ekstrakte edildi ve toplam fenolik içerikleri (TPC) Folin-Ciocalteu yöntemi ile değerlendirildi. Yüksek TPC (sulu < MeOH < EA) gösteren *B. spergulifolius* ekstraktlarının olgun 3T3-L1 adipositlerindeki sitotoksik etkisi MTT asay ile değerlendirildi ve hücre canlılığında %50 azalmaya neden olan ekstrakt konsantrasyonları (IC₅₀) belirlendi. MeOH, EA ve Sulu ekstraktlarının IC₅₀ konsantrasyonları sırasıyla 305.7 ± 5.583 µg/mL, 567.4 ± 3.008 µg/mL ve 418.3 ± 4.390 µg/mL olarak gözlemlendi ve ileri deneylerde kullanılmak üzere seçildi için. Ölü/canlı hücre asay ile MeOH, EA ve Sulu ekstraktlarının sitotoksik etkisi doğrulanmıştır (sırasıyla, %69.75 ± 1.70, %61.75 ± 1.70, %70 ± 4.24 ve hepsi için P < 0.05). Ayrıca, ekstraktların olgun 3T3-L1 adipositlerinde lipid birikimi üzerindeki etkileri Oil-Red-O boyama testi ile değerlendirildi. Tedavi edilmemiş olgun adipositlere kıyasla ekstraktlar ile tedavi edilmiş olgun adipositlerde lipid birikimi etkili bir şekilde azalmıştır (tümü için P < 0.05). Ayrıca ekstraktların *Bax* ve *Bcl-2* tarafından düzenlenen apoptoz üzerindeki etkisi qRT-PCR ile araştırılmıştır. Tedavi edilmemiş adipositlere kıyasla ekstraktlar, pro-apoptotik *Bax* ekspresyonunu artırarak, anti-apoptotik *Bcl-2* gen ekspresyonunu azaltarak apoptozu önemli ölçüde indükledi (hepsi için P < 0.05). İnsülin direnci ile bağlantılı *Glut-4* ekspresyonu, qRT-PCR, Western-blot ve Immunofluorescence boyama ile değerlendirildi. Buna paralel olarak, tedavi edilmemiş olgun adipositlere kıyasla ekstraktlarla tedavi edilen adipositlerde *Glut-4* ekspresyonu, önemli ölçüde daha yüksekti (hepsi için P < 0.05). Ekstraktlar, çizik yara asay uygulamasından 30 saat sonra hücre göçünü önemli ölçüde baskıladı (hepsi için P < 0.05). Hücre morfolojisi ve hücre çapı faz-kontrast mikroskobu, taramalı elektron mikroskobu (SEM), İmmüno Floresans (antiF-aktin ile) ve Giemsa boyaması ile değerlendirildi. Ekstraktlarla tedavi edilen adipositler kısmen küresel morfolojiyi kaybetti ve tedavi edilmemiş adipositlere kıyasla daha küçük hücre çapı gösterdi (hepsi için P < 0.05).

Sonuç olarak, çalışmamız *B. spergulifolius* ekstraktlarının apoptozu indüklediğini, lipid birikimini azalttığını, yarayı iyileşmesini geciktirdiğini, *Glut-4* seviyesini arttırdığını ve bu yolla DM'de insülin direncinin azalmasına katkıda bulunabileceğini göstermektedir.

Anahtar Kelimeler: *Bolanthus spergulifolius*, diyabet, insulin dinci, 3T3-L1 adipositler, glikoz taşıyıcı tip 4, anti-diyabetik

This work was dedicated to my husband Mustafa Çağrı Derici, my mother Aysel Erdoğan and my father Namık Kemal Erdoğan, who encouraged me to overcome all difficulties by being with me under all circumstances with their endless love, patience and support.

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NOMENCLATURE

DM	: Diabetes Mellitus
T1DM	: Type 1 Diabetes Mellitus
T2DM	: Type 2 Diabetes Mellitus
GDM	: Gestational Diabetes Mellitus
preDM	: preDiabetes

<i>B.sperglifolius</i>	: <i>Bolanthus sperglifolius</i>
OGTT	: Oral Glucose Tolerance Test
hbA1c	: Hemoglobin A1c
FPG	: Fasting Plasma Glucose
PG	: Plasma Glucose
2-h PG	: 2-h Plasma Glucose
GLUT'S	: Glucose Transporter's
<i>GLUT-4</i>	: Glucose Transporter-4
IGF-1	: Insulin-like Growth Factor-1
IRS-1	: Insulin-Receptor Substrate-1
IRS-2	: Insulin-Receptor Substrate-2
SGLT's	: Sodium and Glucose Transporters
FDA	: Food and Drug Administration
EA	: Ethylacetate
MeOH	: Methanol
BSA	: Bovine Serum Albumin
DMSO	: Dimethyl Sulfoxide
IBMX	: Isobutylmethylxanthine
DEX	: Dexamethasone
GAE	: Gallic Acid Equivalents
TPC	: Total Phenolic Content
SPSS	: Statistical Package for the Social Science
IC ₅₀	: Half-maximal (50%) Inhibitory Concentration
BCL-2	: B-cell leukemia/lymphoma 2
TSC	: Tuberous Sclerosis Complex
NOS	: Nitric Oxide Synthase
ERK	: Extracellular Regulated Kinase
SLC	: Solute Carriers
GSK	: Glycogen Synthase Kinase
SEM	: Scanning Electron Microscope

1. INTRODUCTION

Diabetes mellitus (DM) is one of the metabolic disease with chronic hyperglycemia characterized by metabolic outcomes because of insulin effect defect insufficient and/or insulin secretion. Chronic hyperglycemia often causes cardiovascular diseases in DM and various complications on the eye, kidney and nervous system (Cho et al., 2018). DM is etiologically classified into three main groups; type 1 DM (T1DM), type 2 DM (T2DM) and gestational DM (GDM). T2DM is the most common type of diabetes among the patients. T2DM accounts for approximately 90% of the total DM (Saeedi et al., 2019). In T2DM, there is a progressive insulin release defect on the basis of insulin resistance, insulin insufficiency (Hu et al., 2013; Xu et al., 2018).

Global DM prevalence was reported as 425 million in 2017, 463 million in 2019, and 578 million in 2030 and it is predicted to increase to 700 million in 2045 (Cho et al., 2018; Saeedi et al., 2019). The dramatic increase in DM is largely due to T2DM as well as metabolic and endocrine dysfunction involving adipose tissue. Metabolic, dysfunction, changes in endocrine secretions and chronic inflammation of adipose tissue contribute to the development of insulin resistance both locally and systemically (Guilherme et al., 2008). Therefore, it is very important to manage T2DM to improve public health by controlling DM, reducing systemic complications and improving insulin sensitivity of adipose tissue (Raveendran et al., 2018). Moreover, developing drug-resistance mechanisms, toxic effects, and high-cost for current drugs in therapy are still major problems for T2DM (Patel et al., 2012). Therefore, it is very important to investigate new therapeutic approaches for DM and alternative anti-diabetic agents that target molecules in potential signal pathways, which are natural, accessible, costeffective, and have low toxicity (Ye, 2013). Medicinal plants are very significant resources in the research of alternative new drug active ingredients (Bilal et al., 2018).

Caryophyllaceae species are rich in phytochemicals and their major chemical components are phenolic substances (Chandra and Rawat, 2015). Caryophyllaceae is one of the largest families that includes approximately 100 genera and 3.000 species (Hernández-Ledesma et al., 2015; Koç et al., 2019). The family Caryophyllaceae found mostly in the Northern Hemisphere especially in the Mediterranean region, rarely in the Southern Hemisphere

(Hernández-Ledesma et al., 2015; Hamzaoglu et al., 2017; Xu, 2017).

The family Caryophyllaceae have antioxidant, anti-viral, anti-fungal, immunomodulatory, antiinflammatory, anti-diabetic and anti-cancer effects and their ethnomedicinal uses is known (Chandra and Rawat, 2015). It has been determined that Caryophyllaceae crude plant extracts can be an important pharmacological resource in developing potential agents against diabetes in addition to high antioxidant capacity and various bioactive properties (Zengin et al., 2018). New approaches to the prevention/treatment of hyperglycemia have popularized the therapeutic uses of α -amylase and α -glucosidase inhibitors. It has also been reported that Caryophyllaceae species have significant α -glucosidase inhibitory action and are a potential natural source of α -glucosidase enzyme inhibitors in the treatment of DM (Hebi and Eddouks, 2019; Yoon et al., 2019). Moreover, phenolic compounds in plants have inhibition activities on α -glucosidase/amylase enzyme cause to decrease blood glucose level (Rasouli et al., 2017).

Bolanthus (Ser.) Reichb., is one of the genera of the family Caryophyllaceae. *Bolanthus spergulifolius* (*B. spergulifolius*) (Jaub. & Spach) Hub.-Mor., in the genus *Bolanthus* (Ser.) Reichb., is a newly described in 2015 and different from other *Bolanthus* taxa, it grows on volcanic slope, steppe in Central Anatolia (Koç and Hamzaoglu, 2015). Although there are a limited number of studies anti-diabetic effect of the family Caryophyllaceae, there are no systematic studies investigating anti-diabetic effect of *B. spergulifolius*. Therefore, in this study, it was aimed to evaluate the anti-diabetic effects in-vitro of *B. spergulifolius* extracts on 3T3-L1 adipocytes.

2. LITERATURE REVIEW

2.1. Diabetes Mellitus (DM)

The DM commonly referred as DM is a significant public health problem (Das et al., 2018). DM is one of the metabolic disease, characterized by hyperglycemia because of insulin effect defect insufficient and/or insulin secretion. DM is one of the most common endocrine disorders affecting almost 6% of the world's population. The global DM prevalence was reported to be

425 million in 2017, 463 million in 2019, and it is projected to rise to 578 million in 2030 and 700 million in 2045 (Cho et al., 2018; Saeedi et al., 2019). The prevalence of this chronic metabolic disease is on the increase. The increasing prevalence projections show that alternative approaches of preventive and medicinal in DM to improving public-health are urgent needed (Raveendran et al., 2018). This disorder affects the metabolism of fat, carbohydrates, protein, water, and electrolytes, leading to structural changes in tissues of many organ systems in the body, especially those of the vascular system.

DM's symptoms such as weight loss, thirst, and sweet-smelling urine are well known. (Adeghate et. al., 2006).

2.1.1. Epidemiology of Diabetes Mellitus

DM is etiologically classified into three main groups; (T1DM), (T2DM) and gestational DM (GDM) (Mekala and Bertoni, 2020). Approximately 5% of all DM is T1DM, 90% T2DM, and 5% of all DM other subtypes. The prevalence and incidence of DM has been increasing across the world over the past 30 years. DM has become one the most pressing and common issue in the last few decades, with the rising obesity , and is now the seventh leading cause of death in the USA as well as worldwide, with 5.2 million deaths globally attributed to DM, a mortality rate of 82.4 per 100.000, with 252,806 deaths in the USA alone in 2015. (Glovaci, 2019). A study conducted by TURDEP II, in which 26,499 people over the age of 20 participated throughout Turkey, showed that the frequency of T2DM was 13.7% and there was a significant increase in its prevalence (Satman et al., 2013). Although a genetic predisposition to DM is important risk factors for an individual, the increasing prevalence of DM at the population level is probably linked to the global obesity epidemic and demographic shifts in older populations. (Mekala and Bertoni. 2020).

The disease burden related to DM is high and rising in every country, fuelled by the global rise in the prevalence of obesity and unhealthy lifestyles. The aetiological classification of DM has now been widely accepted. The premature mortality morbidity, reduced life expectancy and financial and other costs of DM make it an important public health situation (Forouhi and wareham, 2020).

2.1.2. Etiology of Diabetes Mellitus

T1DM, also called insulin dependent DM. T2DM also called non-insulin dependent DM (Ozougwu et al., 2013). T1DM is caused by an autoimmune-mediated destruction of pancreatic beta cells resulting in the absence of insulin, a key hormone regulating blood glucose. T2DM is the most common type of DM among the patients. T2DM accounts for approximately 90% of the total DM (Saeedi et al., 2019). In T2DM, there is a progressive insulin release defect on the basis of insulin resistance, insulin insufficiency (Hu et al., 2013; Xu et al., 2017). Developing drug-resistance mechanisms, toxic effects, and high-cost for current drugs in therapy are still major problems for T2DM (Patel et al., 2012). Therefore, it is very important to investigate new therapeutic approaches for DM and alternative anti-diabetic agents that target molecules in potential signal pathways, which are natural, accessible, costeffective, and have low toxicity (Ye, 2013). Medicinal plants are very significant resources in the research of alternative new drug active ingredients (Bilal et al., 2018).

The causes of DM are incompletely understood. It is widely accepted that the cause of DM is multifactorial and that both genetic and environmental factors play a contributing role. (Adeghate et al., 2006).

T1DM, previously encompassed by the terms juvenileonset diabetes or insulin-dependent diabetes, results from a cellularmediated autoimmune destruction of the b-cells of the pancreas (American Diabetes Association, 2020). T1DM affects all age groups, with most people diagnosed around the age of 4-5 or in adolescence and early adulthood. The incidence of T1DM is rising al over the world. Across Europe, the average annual increase in the incidence of children under the age of 15 is 3.4%, and the fastest increase is observed in those under the age of 5 (Karvonen et al., 1999). There is a strong association between T1DM and other endocrine autoimmunity (for example, Addison disease) and an increased incidence of autoimmune diseases are seen in family members of T1DM patients.

2.1.3. Diagnostic Criteria of Diabetes Mellitus

In 1997, the first Expert Committee on the Diagnosis and Classification of Diabetes Mellitus revised the diagnostic criteria, using the observed association between fasting plasma glucose (FPG) levels and presence of retinopathy as the key factor with which to identify threshold glucose level (American Diabetes Association, 2020). DM is diagnosed with clinical signs and

symptoms. In line with the recommendations of the National DM Data Group (1979), the DM diagnostic criteria, which were standardized by the World Health Organization (WHO) in 1985, were revised by the American DM Association (ADA) in 1997 and by the WHO in 1998-1999, and the fasting blood glucose level was previously 140 mg/dL. It has been taken up to 126 mg/dL. Thanks to many national and international organizations such as ADA and WHO Japan DM Society (JDS), almost every year, the obtained clinical and literature information is scanned and DM diagnosis criteria are kept up-to-date (The expert committee on the diagnosis and classification of DM, 2004; https://www.turkdiab.org/admin/PICS/files/Diyabet_Tani_ve_Tedavi_Rehberi_2019.pdf).

The diagnosis of DM has evolved from physician recognition of typical symptoms to detection of ambient hyperglycemia and, thence, to the definition of excessive plasma glucose levels after an overnight fast and/or following challenge with a glucose load (oral glucose tolerance test; OGTT), and more recently, by measurement of glycated hemoglobin (A1c) (HbA1c). Modern testing has defined individuals at high risk for the development of DM and pregnant women whose fetuses are at increased risk for morbidity and mortality (Saul et al., 2015).

DM may be diagnosed based on plasma glucose criteria, either FPG value or the 2-h plasma glucose (2-h PG) value during a 75-g (OGTT), or A1c criteria. The same tests may be used to screen for and diagnose DM and to detect individuals with preDM. DM may be identified anywhere along the spectrum of clinical scenarios in seemingly low-risk individuals who happen to have glucose testing, in individuals tested based on DM risk assessment, and in symptomatic patients. Also criteria for the diagnosis of DM are shown in Table 1 (American Diabetes Association, 2014).

Table 1. Criteria for the diagnosis of DM

A1C $\geq$ 6.5%. The test should be done using a method that is NGSP certified and standardized to DCCT testing in a laboratory.*
OR
FPG $\geq$ 126 mg/dL (7.0 mmol/L). Fasting is defined as a lack of caloric intake for at least 8 hours.*

OR
2-h PG ≥ 200 mg/dL (11.1 mmol/L) during an OGTT. The test should be carried out using a load of glucose equivalent to 75g of anhydrous glucose dissolved in a water, as describe by the WHO. *
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random PG ≥ 200 mg/dL (11.1 mmol/L) .

In the absence of definitive hyperglycemia, the criteria should be verified by 1-3 retests. Criteria for the diagnosis of GDM is shown in Table 2 (American Diabetes Association, 2020).

Table 2. Diagnosis and screening of GDM

One-step strategy
Perform a 75-g OGTT, with plasma glucose measurement when patient is fasting and at 1 and 2 h, at 24–28 weeks of gestation in women not previously diagnosed with DM. The OGTT should be performed in the morning after an overnight fast of at least 8 h. The diagnosis of GDM is made when any of the following plasma glucose values are met or exceeded: Fasting: 92 mg/dL (5.1 mmol/L) 1 h: 180 mg/dL (10.0 mmol/L) 2 h: 153 mg/dL (8.5 mmol/L)
Two-step strategy
Step 1: Perform a 50-g GLT (nonfasting), with plasma glucose measurement at 1 h, at 24–28 weeks of gestation in women not previously diagnosed with DM. If the plasma glucose level measured 1 h after the load is $\geq 130, 135,$ or 140 mg/dL ($7.2, 7.5,$ or 7.8 mmol/L, respectively), proceed to a 100-g OGTT.

Step 2: The 100-g OGTT should be performed when the patient is fasting. The diagnosis of GDM is made when at least two* of the following four plasma glucose levels (measured fasting and at 1, 2, and 3 h during OGTT) are met or exceeded (Carpenter-Coustan criteria):

95 mg/dL (5.3 mmol/L)

1 h: 180 mg/dL (10.0 mmol/L)

2 h: 155 mg/dL (8.6 mmol/L)

3 h: 140 mg/dL (7.8 mmol/L)

* The American College of Obstetrics and Gynecologists states that a high value can be used for diagnosis.

2.1.4. Classification of DM

DM includes a group of heterogeneous diseases with increased blood glucose concentrations in common (American Diabetes Association, 2014). The basis of the disruptions in fat, carbohydrate and protein metabolism in DM is the insufficiency of insulin in the target tissues. DM causes chronic hyperglycemia, long-term damage to organs, dysfunction or loss. DM, by affecting many organs such as eyes, kidneys, neural structures, heart and blood vessels, causes physical-psychological-economic damage and low-quality of life in the patient (Bhatt et al., 2012). The classification of DM was originally limited to only two categories called juvenile-onset DM, now known as T1DM, and adult-onset DM, now known as T2DM. This has led to the recognition of more than 50 subcategories caused by various pathogenic mechanisms or other concomitant diseases and syndromes. (Saul et al., 2015) DM can be classified into the following general categories:

T1DM (due to autoimmune-cell destruction, usually leading to absolute insulin deficiency) (Puchulu, 2018), results from an autoimmune attack on the pancreatic islet beta cells, manifest by autoantibodies and T cells reactive with islet cell antigens prior to and after the development of hyperglycemia. When about 80% of beta cells are damaged or destroyed, insulin deficiency creates a risk of hyperglycemia and ketosis. Hyperglycemia causes osmotic diuresis that causes frequent urination, thirst and weight loss (American Diabetes Association, 2014). T1DM is seen in 5-10% of DM patients, and the patients are generally children or young people (Satman et al., 2002; Olokoba et al., 2012).

T2DM, is caused by a combination of insulin resistance and relative insulin insufficiency. Insulin resistance accompanies obesity, a sedentary lifestyle, and advanced age. The pathogenetic factors of type 1 and type 2 DM overlap in many patients, with obesity now prevalent in children and adults. Gestational DM is specific for pregnancy and is a harbinger of future type 2 DM (American Diabetes Association, 2014).

Approximately 90% of all DM cases are T2DM (Davis et al., 2010). In T2DM, there is a progressive insulin release defect on the basis of insulin resistance, relative insulin insufficiency and insulin resistance. In the advanced stages of T2DM, insulin resistance cannot be compensated, and a decrease in insulin secretion occurs as a result of impairment in pancreatic cell reserve and function. T2DM is a complex disease in which environmental and genetic factors are effective in its etiology (Qiu et al., 2014). T2DM can often be accompanied by many metabolic diseases such as obesity, hyperglycemia, neuropia, muscle atrophy, hypertension and plasma lipid anomalies (Bonaldo and Sandri, 2013). T2DM has a long asymptomatic pre-DM period (5-15 years) and different complications may develop. Therefore, the primary goal in DM treatment is to prevent the development of T2DM in highrisk individuals (Satman et al., 2013).

GDM is diagnosed during the second or third trimester of pregnancy. Diabetes was not clearly diagnosed before pregnancy.

Specific types of DM due to other causes; Monogenic DM syndromes such as neonatal DM and maturity-onset DM of the young, diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced DM (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation) (Puchulu, 2018).

2.2. Insulin Signaling

Insulin is an anabolic peptide hormone secreted by the β cells of the pancreas. Acting through a receptor located in the membrane of target cells-major ones being, as well as skeletal muscle and fat but also B cells, brain cells and in fact most cells, where it has pleiotropic effects. Its biosynthesis, secretion, structure and structure-activity relationships are thoroughly reviewed (Meyts, 2016).

The insulin signaling system coordinates systemic growth and development with peripheral and central nutrient homeostasis, fertility and lifespan. This simple statement underscores the importance of insulin action in a powerful way. Indeed, insulin regulates glucose uptake, glycogen synthesis, gluconeogenesis, lipid metabolism, hunger, cell growth and division, gene expression and protein synthesis, and vasodilatation (Beale, 2013).

Also Schematic representation of insulin signaling pathway is shown in Figure 1.

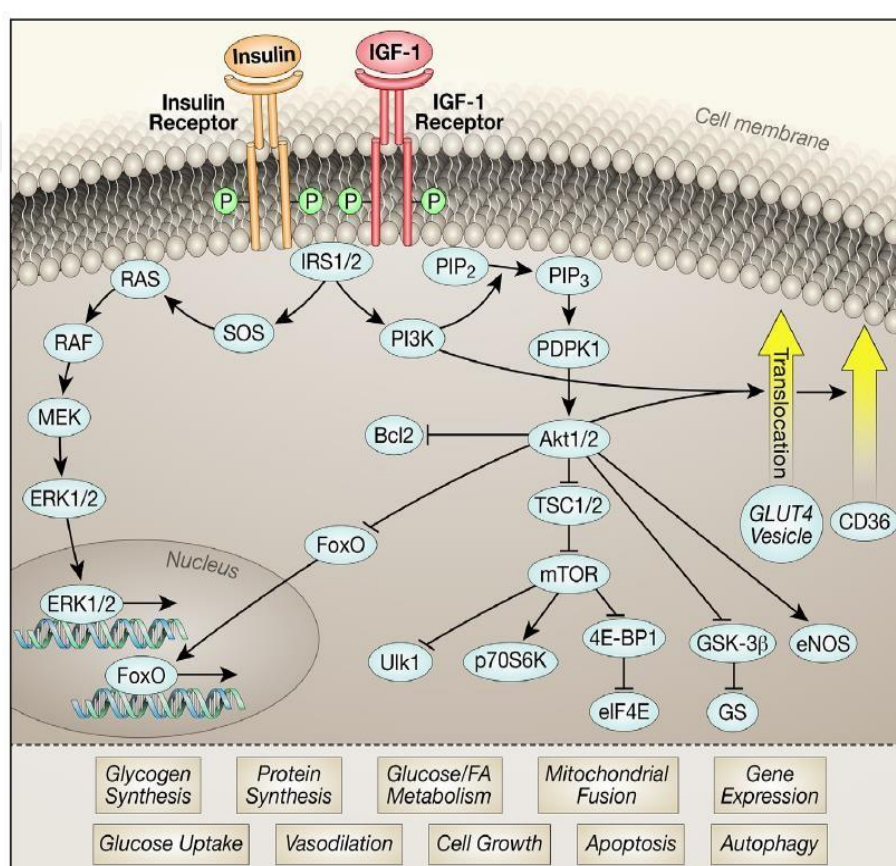


Figure 1. Schematic representation of insulin signaling pathway (Riehle and Abel, 2016).

2.3. Glucose Transport

Glucose is an essential fuel and an important metabolic substrate in mammals. It is obtained directly from diet, mainly after hydrolysis of digested disaccharides and polysaccharides, and by synthesis from other substrates in organs such as the liver. Glucose plays an important role in cellular homeostasis and supporting intermediary metabolism. Since plasma membrane is impermeable to glucose, its cellular uptake is mediated by two distinct processes via specific

glucose transporter proteins that belong to the family of solute carriers (SLC); the SLC2 family members, GLUTs (glucose transporters), are sodium-independent facilitators of the glucose transport, whereas the SLC5 family members, SGLTs (sodium and glucose transporters) mediate the secondary-active sodium- glucose cotransport (Wood and Trayhurn, et al., 2013).

2.3.1. GLUT-4 Translocation

GLUT-4 is one of 14 members of the GLUT/solute carrier family 2 (SLC2A) family of facilitative transmembrane hexose/myo-inositol transporters (i.e., paradoxically, not all of these transport glucose) (Govers, 2014). GLUT4 is regulated by its intracellular localization. In the absence of insulin, GLUT-4 is efficiently retained intracellularly within storage compartments in fat and muscle cells. Upon insulin stimulation (and contraction in muscle), GLUT-4 translocates from these compartments to the cell surface where it transports glucose from the extracellular milieu into the cell. GLUT-4 is mainly expressed in adipose tissue and adult tissues showing insulin stimulated glucose transport, such as skeletal and cardiac muscle. Low levels are also expressed in fetal rat brain. GLUT-4 involves unique sequences in its C- and N-terminal cytoplasmic domains that direct its characteristic membrane trafficking capability. Transport of glucose in insulin sensitive tissues has gained great importance due to the importance of this process in eliminating whole body glucose in an adult. The transport phase is the rate limiting for fat and intramuscular glucose uptake under most conditions (Simmons, 2017).

2.4. Current Approaches to DM Treatment

The most basic treatment component in T2DM is lifestyle change. However, since hypoglycemia and glycemic fluctuations in T2DM can increase mortality, especially in patients with high cardiovascular-disease-risk, drug therapy is very critical. The first choice in treatment is Metformin, an insulin sensitizing agent, and α -amylase and α -glucosidase inhibitors, thiazolidinediones, sulfanylureas, meglitinides are other anti-diabetics used in the treatment (The expert committee on the diagnosis and classification of DM, 2004; Raveendran et al., 2018). https://www.turkdiab.org/admin/PICS/files/Diyabet_Tani_ve_Tedavi_Rehberi_2019.pdf.

Developing drug-resistance mechanisms, toxic effects, and high-cost for current drugs in therapy are still major problems for T2DM (Patel et al., 2012). Therefore, it is very important to investigate new therapeutic approaches for DM and alternative anti-diabetic agents that target molecules in potential signal pathways, which are natural, accessible, cost-effective, and have

low toxicity (Ye, 2013). Drugs in T2DM treatment are quite costly, have common side effects, and lose their effectiveness over time (Dey et al., 2002; Patel et al., 2012).

2.5. Caryophyllaceae

2.5.1. Caryophyllaceae Family

Caryophyllaceae is one of the largest families that includes approximately 100 genera and 3000 species (Hernández-Ledesma et al., 2015; Koç et al., 2019). The family Caryophyllaceae found mostly in the Northern Hemisphere especially in the Mediterranean region, rarely in the Southern Hemisphere (Hernández-Ledesma et al., 2015; Hamzaoglu et al., 2017; Xu et al., 2017). This family is popularly known as the pink family or carnation family. Caryophyllaceae species are rich in phytochemicals and their major chemical components are phenolic substances. The family Caryophyllaceae have antioxidant, anti-viral, anti-fungal, immunomodulatory, anti-inflammatory, anti-diabetic and anti-cancer effects and their ethnomedicinal uses is known (Chandra and Rawat, 2015). It has been determined that Caryophyllaceae crude plant extracts can be an important pharmacological resource in developing potential agents against DM in addition to high antioxidant capacity and various bioactive properties (Zengin et al., 2018). New approaches to the prevention/treatment of hyperglycemia have popularized the therapeutic uses of α -amylase and α -glucosidase inhibitors. It has also been reported that Caryophyllaceae species have significant α -glucosidase inhibitory action and are a potential natural source of α -glucosidase enzyme inhibitors in the treatment of DM (Hebi and Eddouks, 2019; Yoon et al., 2019). Moreover, phenolic compounds in plants have inhibition activities on α -glucosidase/amylase enzyme cause to decrease blood glucose level (Rasouli et al., 2017). The Caryophyllaceae family is mainly centered in the Mediterranean area. The family exhibits great diversity in the habitat and growth form there. Plants of the family are erect, prostrate, annual or perennial herbs or small shrubs, and few species (*Sanctambrosia* spp.) are larger shrubs or small trees. The family Caryophyllaceae is well known for ornamental flowering plants such as

Dianthus chinensis (Pink), *Dianthus barbatus* (Sweet William), *Gypsophila* spp. (Baby's Breath), *Agrostemma* spp. (Corn Cockle), *Saponaria* spp. (Soapwort), *Lychnis* spp. (Fire Pink), and *Silene* spp. (Campions) which form a major fraction of world's cut flower trade (Chandra and Rawat, 2015).

Habit:

Most of them are annual, while some of them are perennial herbs. Certain small shrubs, e.g., some species of *Acanthophyllum* are also found in the warmer parts of the world.

Stem:

The stem is erect, branched, green, herbaceous, solid and mostly swollen at the nodes.

Leaves:

The leaves are simple, opposite, entire and exstipulate.

Inflorescence:

The inflorescence is cymose. Usually it is a dichasium which later on becomes a dichasial cyme ending in a monochasial cyme. This is known as cincinnus inflorescence which is characteristic of the family. In certain cases the flowers are arranged in racemes. In some of genera, e.g., *Arenaria*, *Githago*, etc., the flowers are solitary.

Flower:

The flowers are pedicellate, actinomorphic, usually hermaphrodite and pentamerous, but rarely unisexual or tetramerous. They are regular, complete and hypogynous. Each typical flower bears five whorls, each consisting of five members, such as, five sepals, five petals, two pentamerous whorls of stamens, five carpels, five styles and five double rows of ovules and sometimes five partition walls in the basal part of the ovary.

Calyx:

It is composed of five and very rarely of four sepals. The sepals may be free or united together into a tube. They are usually persistent with membranous margins. The aestivation is imbricate (quincuncial).

Corolla:

It is composed of five and rarely of four petals. The petals are always free (polypetalous). The petals are mostly notched, sometimes bifid, e.g., *Stellaria media*. Usually the aestivation is imbricate.

Androecium:

Usually it consists of ten stamens. Sometimes the number of stamens reduces to eight, five, four, three or even one. They are polyandrous, obdiplostemonous, i.e., the stamens are arranged in two whorls of five each, the stamens of the outer whorl are seen to be opposite the petals and of inner whorl alternating the petals. The stamens are hypogynous and occasionally perigynous. The anthers are two-lobed and dehisce longitudinally. Sometimes the petaloid staminodes are present. In *Spergula*, the five or ten stamens are found to be arranged on a perigynous disc.

Gynoecium:

It consists of two or five carpels; syncarpous. The styles are free. The ovary is superior and unilocular; the ovules are many, campylotropous and arranged on a central column. The placentation is free-central which is most characteristic of the family. The number of carpels corresponds to the number of styles and stigmas. In *Stellaria*, the number of carpels is reduced to three.

Fruit:

Generally the fruit is an unilocular capsule, e.g., *Stellaria*, *Arenaria*, *Spergula*, etc. In some cases the fruit, may be an achene or a nut, e.g., *Herniaria*, *Dysphania*, *Scleranthus*, etc.

Seeds:

The seeds are small and endospermic. The embryo is curved in the endosperm. Sometimes the funicle is conspicuous. They are dispersed by censer mechanism.

Pollination:

It usually takes place by means of insects (i.e., entomophily) (Gupta et al., 2015).

2.5.1.1. *Bolanthus* sp.

Bolanthus (Ser.) Reichb., is one of the genera of the family Caryophyllaceae. *B. spergulifolius* (Jaub. & Spach) Hub.-Mor., in the genus *Bolanthus* (Ser.) Reichb., is a newly described in 2015 and different from other *Bolanthus* taxa, it grows on volcanic slope, steppe in Central Anatolia (Koç and Hamzaoglu, 2015). Also it is different from the species of *Acanthophyllum*. Because its fruit is 8-28-seeded, dehiscent by valves or teeth, leaves, bracts, and calyx never spiny, stamens included calyx. *Bolanthus* sp. involve six species one of which is represented by 2 varieties in Flora of Turkey. As a result of recent studies, 2 and 1 subspecies (*B. creutzburgii* Greuter subsp. *zaffranii* Phitos), have been added to this genus. Anatolia is an important center for *Bolanthus* and 8 species grows in Turkey (Koç, et al, 2019). There are a limited number of studies anti-diabetic effect of the family.

2.5.1.2. Phytochemical and Biological Activity of *Bolanthus* sp.

Medicinal plants are a very important resource for the discovery of new drug agents (Bilal et al., 2018). Today, phytochemical, in-vitro and in-vivo studies are carried out to investigate the anti-diabetic potential of many medicinal plants (Torres and Suarez, 1980; Jouad et al., 2000).

Plant-derived phytochemicals for chronic, complex, and inflammatory infection diseases preventive and/or therapeutic agents has an important potential in the development of, and phytochemical (Hai, 2004; Molyneux et al., 2007). Since modern medicine today relies on natural products, their molecules and derivatives, advances in the scientific and technological field have increased interest in the study of the therapeutic effectiveness, associated molecular interactions, biochemical reactions and structural diversity of these natural products (Mathur and Hoskins, 2017). Phytochemicals obtained from plants; It is known to play an important bioactive role in anti-mutagen, anti-carcinogen anti-inflammatory, anti-diabetic, anti-oxidant and free radical removal (Sun, 1990). The results of research conducted with plants with antidiabetic potential show that the bioactive property that helps control hyperglycemia by inhibiting the α -glucosidase enzyme is caused by phytochemicals such as alkaloids, phenolic compounds, flavonoids, terpenoids, saponins, xanthons, tannins (Hunyadi et al., 2012; Keshari et al., 2016). Anti-diabetic activity of alkaloids isolated from various plants has been reported in in-vitro and in-vivo studies (Costantino et al., 2003; Gaikwad et al., 2014; Agrawal et al., 2013). However, tannins have been reported to inhibit adipogenesis by significantly increasing intracellular glucose uptake in mature 3T3-L1 adipocytes in vitro and anti-hyperglycemic effect in in-vivo DM rats (Muthusamy et al., 2008; Kumari and Jain, 2012).

Ethnobotanical data show that more than 800 medicinal plants are used in the treatment of DM (Alarcon-Aguilara et al., 1998). The hypoglycemic activity of many of these plants has been evaluated and confirmed in different animal models (Torres and Suarez, 1980; Jouad et al., 2000). Along with national organizations, international organizations such as the US Food and Drug Administration (FDA), the International Conference on Compliance (ICH) and the United States Pharmacopoeia (USPC) set standards for the suitability of plant extracts for therapeutic use by establishing various guidelines. Today, the official FDA page has published lists showing that various plant and plant extracts are suitable for anti-oxidant, antidiabetic and anti-inflammatory purposes (<https://www.fda.gov>). Most plant species are protected for this purpose worldwide and their bioactive properties are being investigated (Sasidharan et al., 2011). Currently, there are 505.630 publications in the literature review with “DM”, 255.146 in “DM Medicine” and 7.713 publications in “DM Medicinal Plant” (<https://apps.webofknowledge.com>). Considering the literature studies, the current interest is on the discovery of accessible, cost-effective, low-toxicity natural new drugs that target molecules

in potential signal pathways in addition to protocols used in the treatment of DM (Bilal et al., 2018).

Phytochemical studies have shown that Caryophyllaceae species are rich in compound content and contain bioactive metabolites such as saponins, flavonoids, phenolic acids, phytoecdisteroids and oligosaccharides (Lawrence, 1951; Bittrich, 1993; Golea et al., 2017). The Caryophyllaceae species are widely used for medicinal purposes by various ethnic communities. Species in the family Caryophyllaceae; traditionally, cough, eye and skin problems, dysentery, headache, heart disease, DM, kidney stones, cataracts, epilepsy, asthma, fever, hypertension, and menstrual irregularities such as is used to treat diseases (Eddouks et al., 2003; Fernandez et al., 2003; Macia et al., 2005; Purkayastha et al. 2005; Chandra and Rawat, 2015). Biological studies have shown that plant extracts belonging to Caryophyllaceae species are anti-fungal (Ameziane et al., 2007), anti-viral (Barakat et al., 2010), antiinflammatory (Cordell et al., 1977), anti-oxidant (Karamian and Ghasemlou, 2013) antidiabetic (Eddouks et al., 2003; Oyedemi et al., 2009; Hu et al., 2013; Hebi and Eddouks, 2019; Yoon et al., 2019), anti-tumor (Khan et al., 2010), anti-bacterial (Bonjar, 2004), diuretic (Al-Quran, 2008), analgesic (Angmo et al., 2012). In a study conducted in 2018, the antioxidant capacity and anti-inflammatory properties of the plant extracts of *Silene salsuginea*, which is a species of Caryophyllaceae in our country, were analyzed by various in-vitro and in-silico methods. It has been reported that Caryophyllaceae species are the natural source of biologically active compounds for phyto-pharmaceuticals and can be used in the treatment of chronic diseases such as inflammation, cancer and DM. However, it has been reported that more analytical experiments are needed by using systematic in-vitro and in-vivo models to investigate its biological effects and effective doses (Zengin et al., 2018b). In a study conducted in 2013, methanol extracts of *Dianthus chinensis* and *Acalypha australis* L. species from the Caryophyllaceae family induced apoptosis in cancer cell lines compared to normal healthy cells and showed therapeutic effect by reducing SP1 expression. In addition, this study highlighted that Caryophyllaceae species have potential bioactive natural products that can be used to treat cancer (Shin et al., 2013).

Corrigiola telephiifolia, *Argania spinosa*, *Nees* and *Cinnamomum tamale* with garcinia extract derived from in vitro studies of Caryophyllaceae species to cambogi extremely rich as a source

of phenolic compounds, anti-inflammatory, anti-lipolytic and/or anti-adipogenic, anti-oxidant activity and in vivo studies after treatment of diabetic rats a significant decrease in plasma cholesterol level was observed and a strong anti-hyperglycemic effects have been reported (Koshy et al., 2001; Dhulasavant et al., 2010; Hebi and Eddouks, 2018; Hebi et al., 2018). However, its potential as a powerful anti-diabetic agent has been shown in in-silico, invitro and in-vivo studies (Zhang et al., 2016; Uysal et al., 2018; Zengin et al., 2018; Yoon et al., 2019). These studies show that the use of the Caryophyllaceae family for medical and therapeutic purposes has a high potential.

Plant extracts of the Caryophyllaceae species, including *Gypsophila paniculata* L., *Gypsophila trichotoma* Wend., *Saponaria officinalis* L., and *Dianthus sylvestris* Wulffen, on human monocytic THP-1 and alveolar macrophage NR8383 cell lines, thereby increasing the activity of the caspase-3 It has been determined that Caryophyllaceae crude plant extracts can be an important resource in the development of potential agents that can be used in the treatment of various diseases underlying oxidative stress and chronic inflammation in the pathogenesis, especially cancer (Gevrenova et al., 2014). Izoorientin-2-O α L-arabinopyranosyl (IOA) isolated from *Gypsophila elegans* has been shown to be effective in reducing liver damage and fibrosis in an alcohol-induced rat model, which should be developed as a new drug to treat liver-induced fibrosis with its antioxidant potential (Huang et al., 2012). In addition, in a study conducted in in-vitro / in-vivo mice, it was reported that *Gypsophila trichotoma* showed hepatoprotective and antioxidant activity against CCl₄-induced liver cell injury. It has been reported that triterpenoid saponins isolated from *Gypsophila oldhamiana* significantly inhibit the α -glucosidase enzyme and are a potential source of natural α -glucosidase inhibitors in the treatment of DM (Luo et al., 2008). New current approaches developed for the prevention/modulation of hyperglycemia reveal the importance of the therapeutic use of α -amylase and α -glucosidase inhibitors. Kasabri et al. Reported that Caryophyllaceae species can improve glucose homeostasis by significantly delaying carbohydrate digestion. However, they are highly promising as therapeutic agents for improvements in impaired peripheral carbohydrate tolerance and are an important natural resource for the discovery of antidiabetic therapeutics (Kasabri et al., 2011). In an in-vitro study by Lee et al., *Melandrii Herba* extract from the same family activates the intrinsic apoptotic pathway, down-regulation of *Bcl-2* by *Caspase-3*, up-regulation of *Bax* and activation of A mitogen-activated protein kinase (MAPK) have been reported to protect human neuroblastoma

SH-SY5Y cells from H₂O₂-induced-neurotoxicity. In addition, in-vivo study data from a scopolamine-induced cognitive impairment induced mouse model showed that the *Melandrii herba* extract can alleviate symptoms, reporting that the herb is worthy of further investigation as a complementary or even alternative product for preventing and treating diseases involving neurotoxicity and neurodegeneration (Lee, et al., 2017).

2.5.1.3. *Bolanthus spregulifolius*

B. spregulifolius (Jaub. & Spach) Hub.-Mor., in the genus *Bolanthus* (Ser.) Reichb., is a newly described in 2015 and different from other *Bolanthus* taxa, it grows on volcanic slope, steppe in Central Anatolia (Koç and Hamzaoglu, 2015). Although there are a limited number of studies anti-diabetic effect of the family Caryophyllaceae, there are no systematic studies investigating anti-oxidant and anti-diabetic effect of *B. spregulifolius*. Therefore, in this study, it was aimed to evaluate the anti-diabetic effect of extracts of *B. spregulifolius* on 3T3-L1 adipocytes.

Caryophyllaceae, there are no systematic studies investigating anti-oxidant and anti-diabetic effect of *B. spregulifolius*. Therefore, in this study, it was aimed to evaluate the anti-diabetic effect of extracts of *B. spregulifolius* on 3T3-L1 adipocytes. The taxonomy of *B. Spregulifolius* is shown in Table 3.

Table 3. The taxonomy of *B. Spregulifolius*

Realm:	Plantae
Subalem:	Tracheobionta
Branch:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Caryophyllidae
Team:	Caryophyllales
Family:	Caryophyllaceae
Subfamily:	Caryophylloideae
Tribus:	Caryophylleae
Genus:	<i>Bolanthus</i>
Species:	<i>spregulifolius</i>

2.6. 3T3-L1 Cell Line as In-vitro Model

In many studies, cell lines are used to investigate the cytotoxic efficacy of plant extracts. Two in-vitro models have been used to understand the mechanisms in adipocyte proliferation, differentiation and adipokine secretion from studies over the past 25 years. The first is cell lines, which have the ability to differentiate into adipocytes such as 3T3-L1. The second is multipotent stem cells, which have the capacity to transform into different cell lines such as fat, novelties and bones. 3T3-L1 is a preadipocyte cell line produced from mouse *Mus musculus*. 3T3-L1 cells have a fibroblast-like morphology and have the ability to differentiate into adipocyte-like phenotype under favorable conditions, so they are quite useful for investigating diabetes pathogenesis (Green and Kehinde, 1975; Akinrinde et al., 2018; Hu et al., 2018). 3T3-L1 preadipocyte differentiation is stimulated by endocrine and autocrine factors. These factors induce the synthesis and activation of transcription factors in adipogenesis. 3T3-L1 preadipocytes by induction go through many cellular division processes called mitotic clonal expansion, genes in triglyceride metabolism are activated by peroxisome proliferator and lipid droplets are formed in the cytoplasm by differentiating into mature adipocyte. In adipocyte differentiation, mitochondrial activity becomes important and requires a large number of mitochondria in the maturation process (De Pauw et al., 2009). Small adipocytes in the maturation process are sensitive to insulin and synthesize adiponectin and adipokine (Koh et al., 2007; De Pauw et al., 2009). Mature and large adipocytes, develop resistance to insulin due to a lack of mitochondria. By activating adipogenesis and Lipogenesis at low levels of mitochondrial ROT, it increases adipocyte count-size and blocks high ROT differentiation (Caron-Debarle et al., 2010). Mature adipocyte is fat cell specialized in fat synthesis and storage. In response to Hormonal stimuli, adipocytes store energy by lipid accumulation. They appear swollen under a microscope and filled with lipid droplets. When mesenchymal adipocyte precursor cells are cultured in a differentiation medium consisting of insulin, dexamethasone, and methylisobutylxanthin, the expression of adipocyte markers has been shown to increase (Ringold, 1988; Spiegelman, 1993; Cornelius, 1994; Flier, 1995; akinrinde et al., 2018; Hu et al., 2018). In 3T3-L1 adipocytes, ROT occurs by lipid accumulation, invitro this model indicates that adipose tissue is an important source for ROT (Bravo, 1998).

Adipocytes obtained by 3T3-L1 cell line differentiation are a useful in-vitro model in order to determine the effect of *B. spergulifolius* extracts on lipogenesis pathways in our study.

Therefore, as part of our study, the effective doses of *B. spergulifolius* extracts will be determined by differentiating preadipocyte 3T3-L1 cells into mature adipocytes and further research will be carried out.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant

B. spergulifolius used in this study was collected from Gediz, Murat Mountain (Turkey, Kütahya province) during the flowering season in the year 2019 (Figure 2). Identification and authentication of the plant material were carried out by Dr. Murat Koç, taxonomist, from Department of Traditional, Complementary and Integrative Medicine Graduate School of Public Health, Ankara Yıldırım Beyazıt University, Turkey. Voucher specimen of *B. spergulifolius* (No. Koç2043&Hamzaoglu) were deposited in the herbarium of Biology Department of Bozok University, Turkey (Hamzaoglu et al., 2017). The air-dried plant materials under shade were ground in a coffee grinder (Kruips 75, France) (Zheleva-Dimitrova et al., 2018).



Figure 2. Morphological image of *B. spergulifolius*

3.1.2. 3T3-L1 cell line

The preadipose cell line 3T3-L1 was originally developed by clonal expansion from murine Swiss 3T3 cells. Because of its potential to differentiate from fibroblasts to adipocytes, the cell line has widely been used in more than 5.000 published articles on adipogenesis and the biochemistry of adipocytes (Zebish, et. al., 2012). The 3T3-L1 cell line is a well-established and commonly used in vitro model to assess adipocyte differentiation (Chao 2007).

3T3-L1 fibroblast rat embryonic white fat precursor cell clone-type cells and differentiation of these cells to accumulate lipid droplets within the cell when the medium is applied to the property, has a feature to win the adipocyte phenotype (Green and Meuth, 1974; Kallen and Lazar, 1996). For this reason, 3T3-L1 (CRL-173) was used in our study and the cells are sourced from the American Type Culture Collection (ATCC, Manassas, VA). Cells and receptor expression has been well characterized, and many products such as cellular genes that are expressed specific properties are well known (<https://www.lgcstandards-atcc.org>).

3T3-L1 cell line is shown in Figure 3.



Figure 3. 3T3-L1 cell line

3.1.3. Chemicals

Insulin, Isobutylmethylxanthine (IBMX), Dexamethasone (DEX), Bovine serum albumin (BSA), Glutaraldehyde, Ethylacetate (EA), Methanol (MeOH), Ethanol, Dimethyl sulfoxide (DMSO), Phosphate buffered saline (PBS), $+/+$ PBS (containing Ca^{++} and Mg^{++}), 4',6-Diamidino-2-phenylindole (DAPI), Formalin, Isopropanol, 2-propanol, Chloroform TRIZOL, DEPC water, Lipid (Oil Red O) Staining Kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Boric acid, Tris, Thylenediaminetetraacetic acid, Sodium dodesylsulfate, Tris, Glycine, Mercaptoethanol, RIPA buffer, Protease inhibitor cocktail, Skim milk powder, Folin-Ciocalteu, Sodium carbonate, Gallic acid, Triton X-100, Tween 20, Paraformaldehyde, Hexamethyldisilazane (HMDS), Giemsa were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin, Streptomycin, L-Glutamine were obtained from Hyclone (GE Healthcare, USA). Calcein-Acetomethoxy (C-AM) (ab141420), Ethidium homodimer-1 (Eth1) (ab145323), anti-Glut-4 (ab35826 and ab654), anti- β -Actin (ab8226), Goat Anti-mouse

IgG secondary antibody (ab205719), Goat Anti-Mouse IgG H&L (Alexa Fluor®488) (ab150113) Phalloidin-iFluor 488 Reagent (ab176753) and BrightMount/Plus were purchased from Abcam (Cambridge, UK). Quick Start Bradford Protein Assay Kit 1, 2x Laemmli sample buffer, 10x Tris/Glycine/SDS buffer, Immun-Blot PVDF membrane, ECL reagent were purchased from Bio-Rad (Hercules, USA).

3.1.4. Instruments

Weighing (Denver Instruments, Timberline, Model-TP-214, US)

Ultrasonic Bath (Daihan, Model-WUC-06AH, USA)

Rotary Evaporator (Marshall Scientific, Model-Buchi-R200, HAMPTON, USA)

Invert Microscope (Thermofischer, Leica, Dm1e Led Fluor, Germany)

Class II Biohazard Safety Cabinet (Nuaire, LabGard NU-540, US)

CO₂ Incubator (Nuaire, NU-5800, USA)

Spectrophotometer (Shimadzu, UVmini-1240, Kyoto, Japan)

Spectrophotometer (Thermo Scientific, Nanoprop 2000c, USA)

Centrifuge (Beckman Coulter, GA-10 fixed Angle Rotor, California, USA)

Centrifuge (Hettich, Rotofix 32A, Darmstadt, Germany)

Shaker (Benchmark, Everlast Rocker 247, USA)

Blotting (Bio-Rad, ProSemi-Dry Blotting, USA)

Real-Time PCR (Qiagen, Hilden, Germany)

Thermal Cycler (Agilent, SureCycler 8800 Thermal Cycler, USA) pH/conductivity meter (Mettler TOLEDO)

Ultra-Low Temperature Freezer (Nuaire, NU-99486E, UK)

3.2. Methods

3.2.1. The extraction of *B. spregulifolius*

B. spregulifolius plant extracts were prepared with three different solvents, EA, MeOH and Aqueous: for the preparation of plant extracts, after *B. spregulifolius* is dried in the shade, hanging/laying in the air in suitable conditions; all the above ground parts were pulverized in the mill (Shin et al., 2013; Kim, et al 2012).

3.2.1.1. The fractionation of of *B. Spergulifolius*

For *B. spergulifolius* MeOH extraction; 5 g of dried plant samples was extracted with 100 mL MeOH for 1 h in an ultrasonic bath (Ultracleaner, DH-WUC-06H, Daihan, USA) at 15°C. This proses was replicated until the solvent turned colourless. After the plant extract were filtrated using Whatman No. 1. Then the MeOH was removed from the filtrate of extract by a rotary evaporator (Buchi Rotavapour R-200, Marshall Scientific, Hampton, USA) at 37 °C. Then, the filtrate was stored at -80 °C.

For *B. spergulifolius* EA extraction; 5 g of dried plant samples was extracted with 100 mL EA and for 1 h in an ultrasonic bath (Ultracleaner, DH-WUC-06H, Daihan, USA) at 15°C (10 min intervals in three-times). After the filtration of extracts were performed using Whatman No. 1. The filtrate of extract was concentrated by a rotary evaporator (Buchi Rotavapour R-200, Marshall Scientific, Hampton, USA) at 37 °C. Then, the filtrate was stored at -80 °C.

For *B. spergulifolius* Aqueous extraction; 5 g of dried plant samples was extracted with 100 mL of water for 1 h in an ultrasonic bath (Ultracleaner, DH-WUC-06H, Daihan, USA) at room temperature (10 min intervals in triplicates) and filtered (Whatmann No.1) under vaccum. The filtrate of Aquaeous was frozen at -20 °C and then dried with freeze dryer (BKFD10P, Biobase, China).

The extracts of plant sample were weighed (TP-214 Timberline, Denver Instrument Company, UK) and the yield of all of the extraction was calculated in terms of (w/w) % per g crude extract. The extracts were reconstituted in DMSO at a final-concentration and stored at -20°C under shade until further studies.

3.3. Folin-Ciocalteu method

Total phenolic content (TPC) was determined by Folin-Ciocalteu method (Singleton et al., 1999). 100 µL 10% (v/v) of Folin Ciocalteu reagent was added into 20 µL samples of each plant extract for 5 minutes at room temperature. Finnaly, 80 µL 2% (w/v) of sodium carbonate were added and incubated in the dark for 30 min at 40 °C. The absorbance was measured at wavelength of 765 nm with a spectrophotometer (Uvmini-1240, Shimadzu, Kyoto, Japan).

According to the standard calibration curve, values of TPC for each extract were expressed as gallic acid equivalents (mg GAE/g of dry weight).

3.4. Cell culture

3.4.1. The growth of 3T3-L1

The 3T3-L1 was used in the current study. In experimental studies verified ancestral morphological and molecular traits of the cells they will start with. During the study, experimental-along with the lines from which the studies were conducted, the parent cells would be cultured under normal conditions and used to show that ancestry persisted in morphological and molecular comparisons at all stages of the study.

3T3-L1 were cultured by growth medium; Modified Eagle's Medium Dulbecco of 500 mL (DMEM)/High Glucose (Hyclone, GE Healthcare, USA), 50 mL Fetal bovine serum (FBS) (Hyclone GE Healthcare, USA), 5 mL Penicillin (100 U/mL)-Streptomycin (100 µg/mL) (Hyclone, GE Healthcare, USA) and 5 mL L-glutamine (Hyclone, GE Healthcare, USA) and prepared by adding +4 °C was maintained.

3.4.2. The thawing and freezing of 3T3-L1

For the 3T3-L1 cell line culture, primarily the water bath was adjusted to 37 °C. Medium was left to warm up for 20-30 min. The cell lines were thawed at room temperature after being removed from the stock at -80 °C. The Class 2 biosafety cabinet was cleaned and opened with alcohol and taken with 1000 µL micropipet and gently mixed with 5 mL medium in 15 mL falcon. It was centrifuged for 5 min at 1.100 rpm in macro centrifuge. Supernatant ejected. Cells dissolved in 2 mL clean medium. 3 mL more medium was added on it, T-25 cm² flaska was transferred. Mixed with + on the ground, removed to incubator with 37 °C 5% CO₂ adjustment (Figure 4).

3T3-L1 cells that were surplus at the end of culture operations or during passages were stockpiled for later reuse. Stock solution was prepared before starting the inventory process. the stock solution was always prepared fresh and cold in the form of 90% FBS containing 10% DMSO. It was kept in ice until the appropriate amount of prepared stock solution was used.

Cells to be stockpiled were washed 2 times, primarily with 1X DPBS. Then 2 mL of 0.25% trypsin-EDTA solution was removed from the surface they were holding. After the removed cells were taken as pellets, they were thawed in 5 mL clean medium. The thawed cells were centrifuged for a second time for 3 minutes at 1.100 rpm. The supernatant was ejected after centrifuge. The cells in the Pellet section were thawed in stock solution. Cells in the pellet part were dissolved in stock solution. Afterwards, 106 3T3-L1 cells were transferred to cryovial tubes each 106 in 1 mL. After the transfer, the tubes were first kept at +4 °C for 10 minutes, then at -20 °C for 120 minutes and lastly they were raised to -80 °C and taken into stock.



Figure 4. The cabin where cell culture experiments are done.

3.4.3. The passaging of 3T3-L1

The 3T3-L1 cells was observed under invert microscope (Leica, Dmil Led Fluo, Germany) until they become 70% confluent in Nuaire NU-5800 model incubator with humidified air at 37°C with 10% FBS, 1% Penicillin + streptomycin in DMEM fattening media supported by 1% L-glutamine containing 5% CO₂ (Shimizu et al., 2011). The 3T3-L1 cells were passaged when they were sub-confluent. If the 3T3-L1 cells were 100% confluent, they could not differentiate into mature adipocytes (Figure 5). The growth medium was discarded using micropipettes from the well. After the cells were washed two twice with 1X DPBS (Sigma Aldrich, USA), and then removed with 2 mL of 0.25% Trypsin-Edta Solution (Sigma Aldrich, USA), and incubated at 37°C for 5 minutes. 0.25% Trypsin-Edta Solution was inactivated by 5 mL of DMEM.

The shield cells from the surface they hold were collected with 5 mL medium and then taken into a 15 mL falcon tube and centrifuged for 5 min at 1,100 rpm. The supernatant was ejected after centrifuge. The cells in the pellet section were dissolved with clean medium (Figure 6).

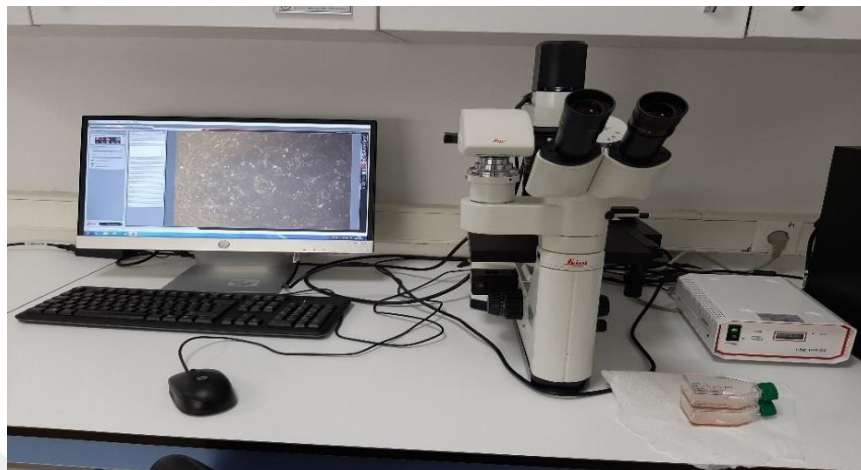


Figure 5. Control of the cells under inverted microscope after sowing.



Figure 6. Passaging cells into flasks.

3.4.4. The counting of 3T3-L1

In all experiments, cell counting was performed with haemocytometer. The cultured 3T3-L1 cells were washed 2 times with 1X DPBS (Sigma Aldrich, USA) and removed from the surface they were clinging to with a 0.25% trypsin-EDTA (Sigma Aldrich, USA) solution. 15 μ L and 0.4% trypan blue (0.5% w/v) dye were then mixed in 0.2 mL ependorf tube and incubated for 5 minutes from thawed cell suspension in clean medium. Then the haemocytometer was put in a 10 μ L cell-trypan blue dye mixture and the lamella was closed on it. The haemocytometer was examined under the inverted microscope and the cell count process was completed (Figure

7). As a result of the counting, the total number of cells and the percentage of cells viability were obtained from the device.

The tripan blue dye the number of living cells and the percentage of viability was calculated by formula (Freshney, 2000).

Living cell ratio (%)= number of cells without dye / total number of cells x 100.

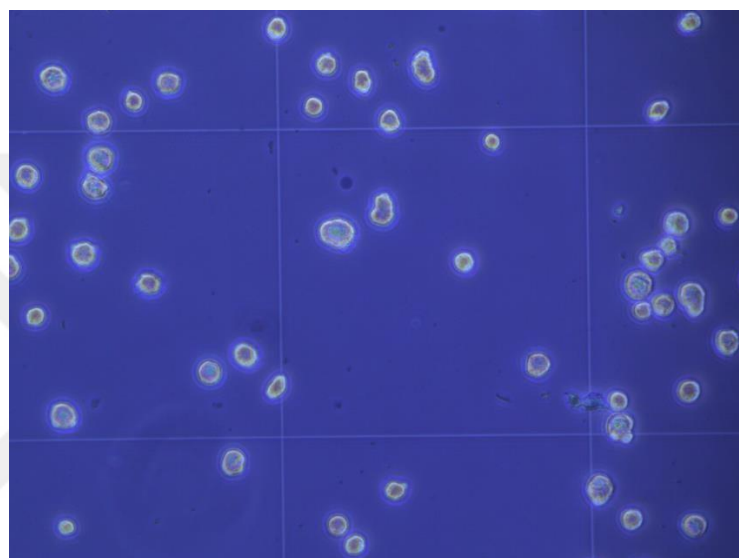


Figure 7. Image of 3T3-L cells stained with 0.4% trypan blue under a haemocytometer inverted microscope (10X).

3.5. The differentiation of 3T3-L1 into mature adipocytes

In order to determine the effective dose of *B. spergulifolius* extracts and to carry out further research, preadipocyte 3T3-L1 cells need to differentiate into mature adipocytes, so differentiation was induced by special media in the cells differentiation media (Figure 8).

According to ATCC protocol, 3T3-L1 cells were cultured at a density of;

8×10^4 cells/well for 6 well-plate (9.6 cm^2)

2×10^4 cells/well for 24 well-plate (1.9 cm^2)

2×10^3 cells/well for 96 well-plate (0.32 cm^2)

3T3-L1 differentiation medium according to ATCC protocol; 10% FBS (Hyclone, GE Healthcare, USA), 4 mM L-glutamine (Hyclone, GE Healthcare, USA), 1% Penicillin+streptomycin antibiotics (Hyclone, GE Healthcare, USA) containing 90% DMEM (Hyclone, GE Healthcare, USA) Dexamethasone (1 μ M; Sigma Aldrich, USA), Insulin (1 μ g/mL; Sigma Aldrich, USA), and Methylisobutylxanthine (IBMX) (0.5 mM; Sigma Aldrich, USA) in the medium by adding was prepared .

3T3-L1 pre-adipocytes (8×10^4 cells/well) was seeded in a 6-well plate and 48 hours later, when the cells become 100% confluent, the media was removed and cultured by fresh differentiation media. Then growth medium was removed and differentiation medium was added. At this stage, the 3T3-L1 detached easily from the well-plate so gentle pipetting and handling was needed.

3T3-L1 adipocyte maintenance medium according to ATCC protocol; 10% FBS (Hyclone, GE Healthcare, USA), 4 mM L-glutamine (Hyclone, GE Healthcare, USA), 1% Penicillin+streptomycin antibiotics (Hyclone, GE Healthcare, USA) containing 90% DMEM (Hyclone, GE Healthcare, USA) Insulin (1 μ g/mL; Sigma Aldrich, USA), and in the medium by adding was prepared.

3T3-L1 was seeded in differentiation medium and 48 hours later, the differentiation medium was removed and added by adipocyte maintenance medium (Figure 9). A fresh adipocyte maintenance medium was added every two days and 12-14 days later the differentiation of precursor adipocytes into mature adipocytes was completed and the morphological differentiation in cells was displayed using an invert microscope (Leica, Dm1100 LED Fluor, Germany) (Akinrinde et al., 2018; Hu et al., 2018) (Figure 10).

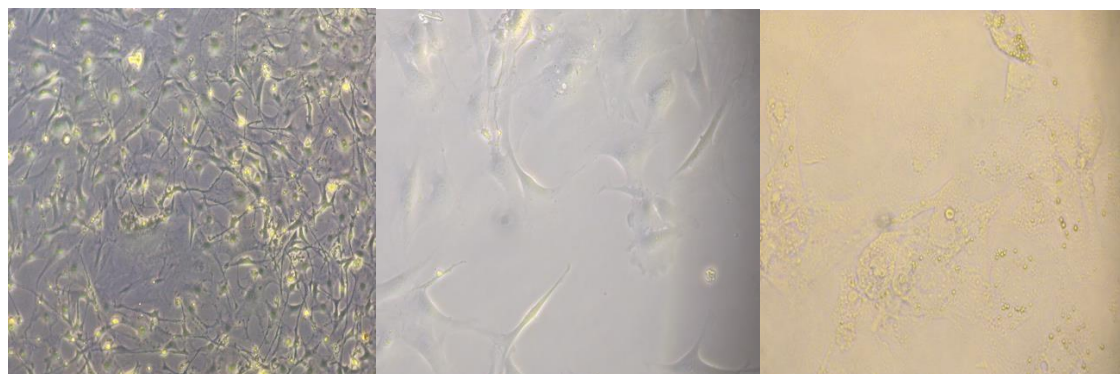


Figure 8. Image of 3T3-L1 pre-adipocyte cells under an inverted microscope (10X, 20X and 40X).

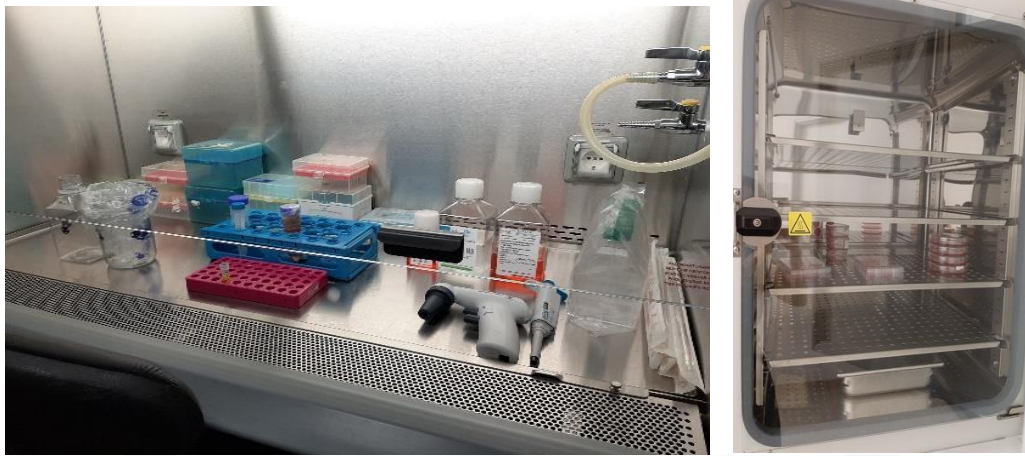


Figure 9. 3T3-L1 adipocyte maintenance.

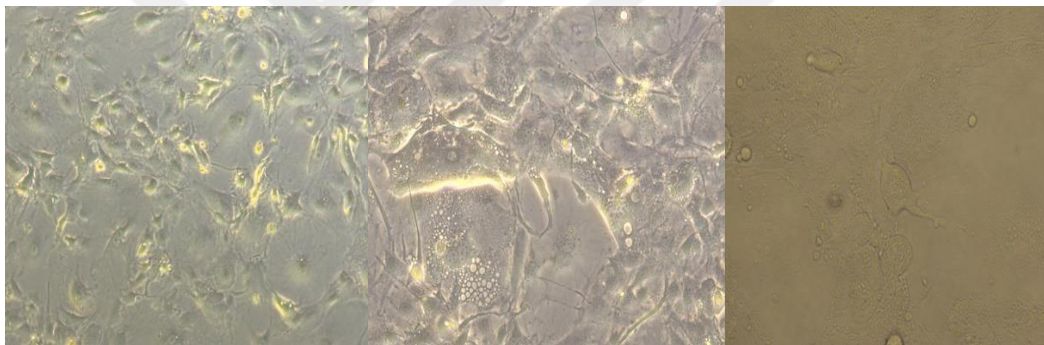


Figure 10. A-B-C) Image of mature adipocyte cells under an inverted microscope (10X, 20X and 40X).

3.6. Growth conditions of 3T3-L1

3T3-L1, an embryonic mouse preadipocyte cell with the capacity to differentiate into adipocytes by chemical induction, is an important in-vitro model for DM studies. 3T3-L1 lines were cultured under appropriate conditions (Figure 11) as the yet undifferentiated preadipocyte 3T3-L1 cells may lose contact inhibition in the intensive culture process, 70% confluent cells were traversed. As cells proliferate fast, they have reached 70% confluency in 48 hours.

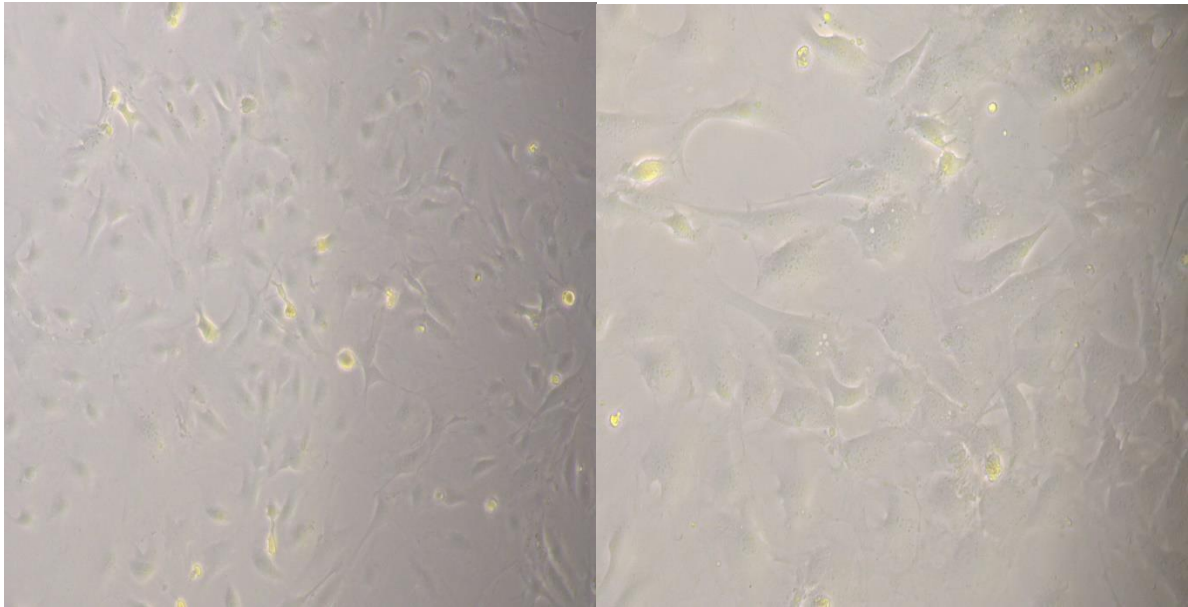
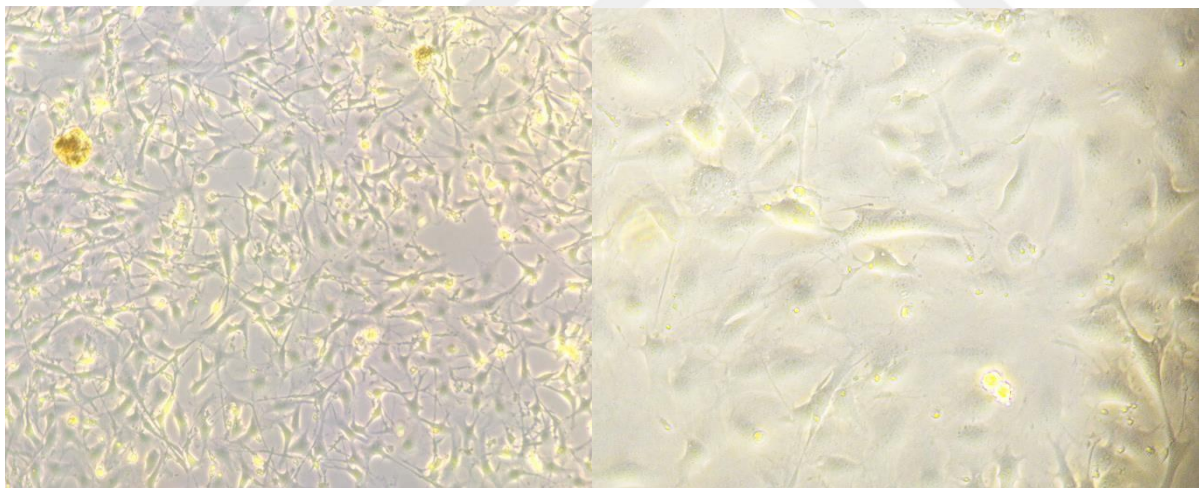


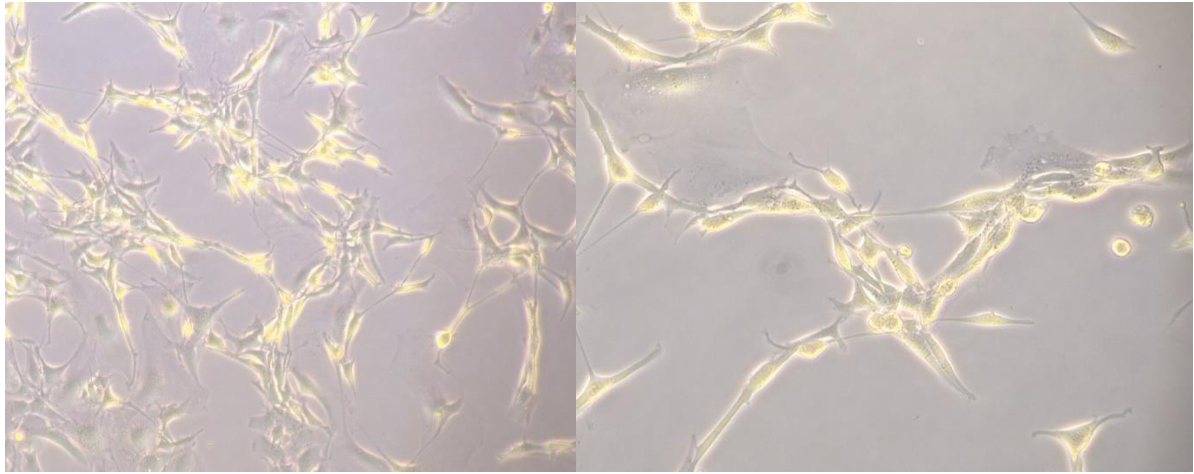
Figure 11. Image of 3T3-L1 preadipocyte cells under an inverted microscope (10X and 20X).

3.7. Differentiation of 3T3-L1

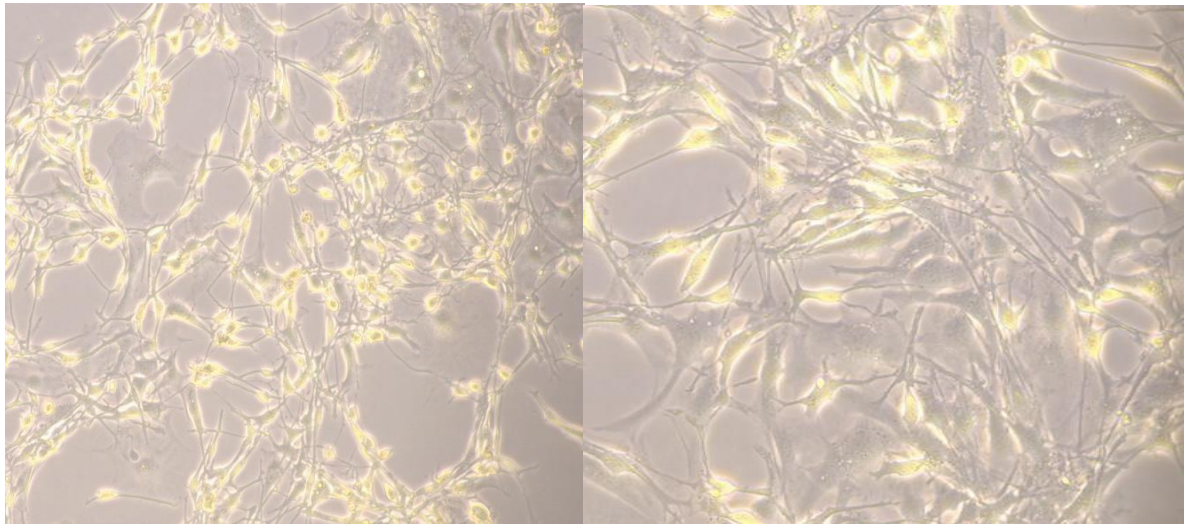
Phase contrast image on the days that the protocol was followed are shown in Figure 12.



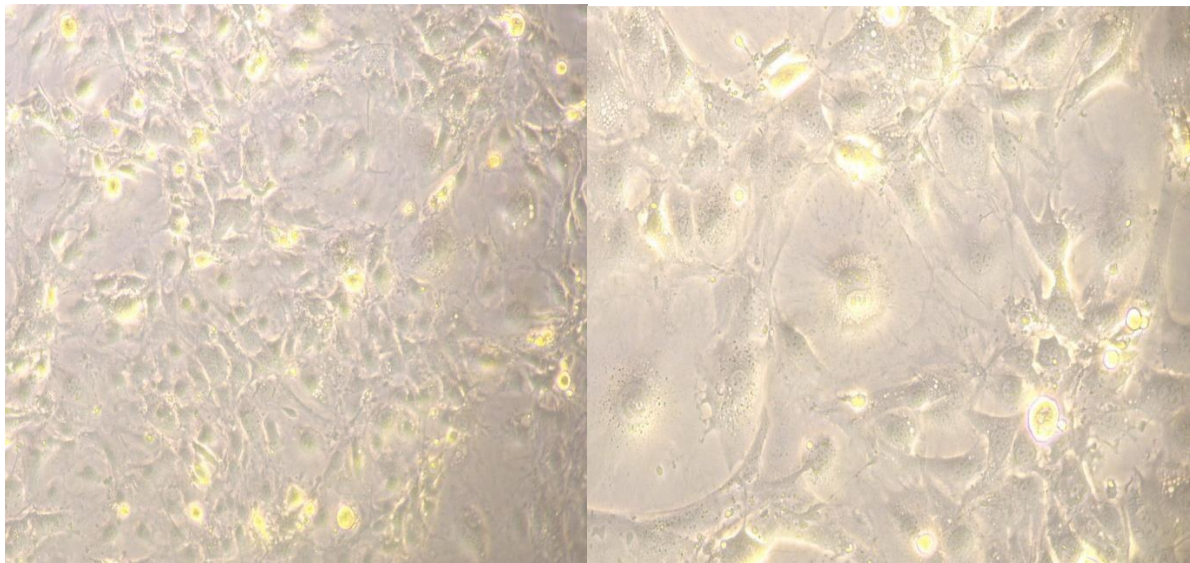
A) Day 0 in differentiation (10X and 20X)



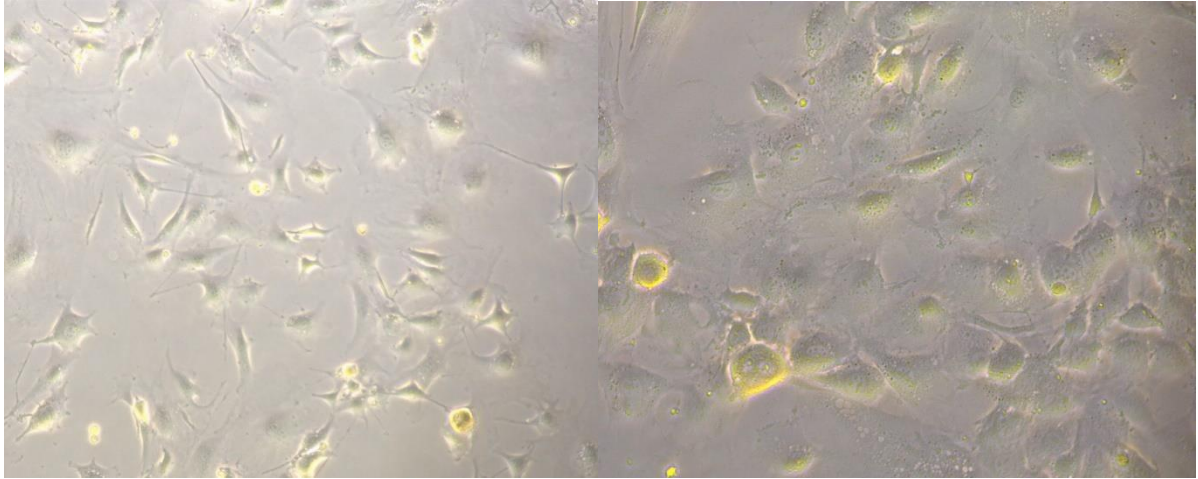
B) Day 2 in differentiation (10X and 20X)



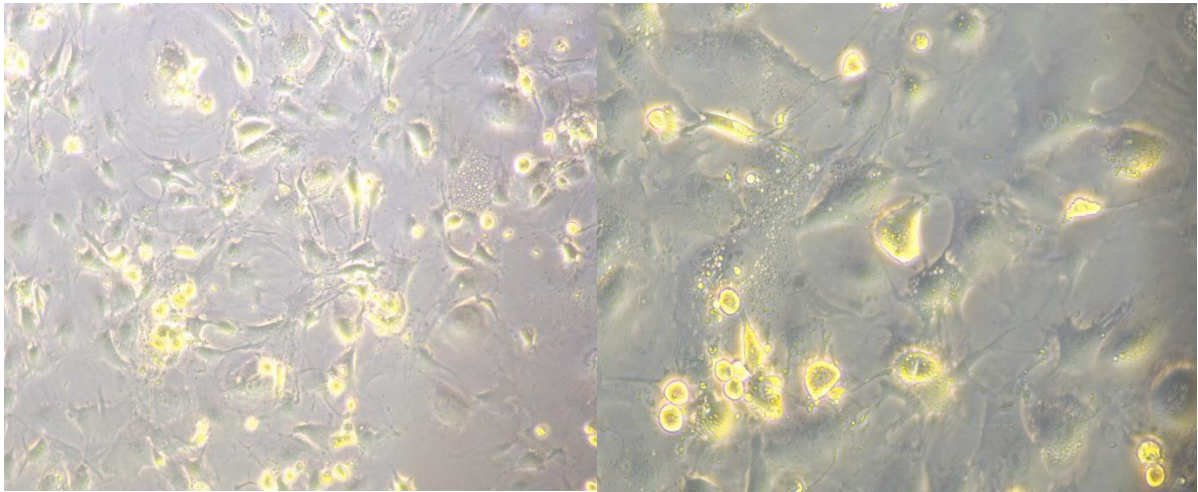
C) Day 2 in differentiation (10X and 20X)



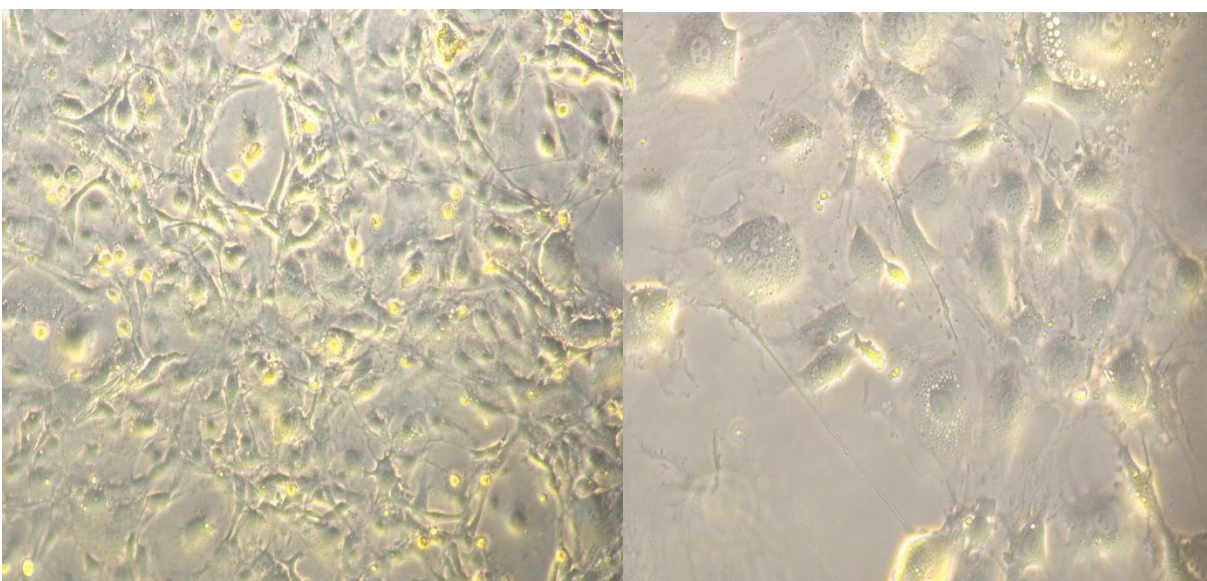
D) Day 6 in differentiation (10X and 20X)



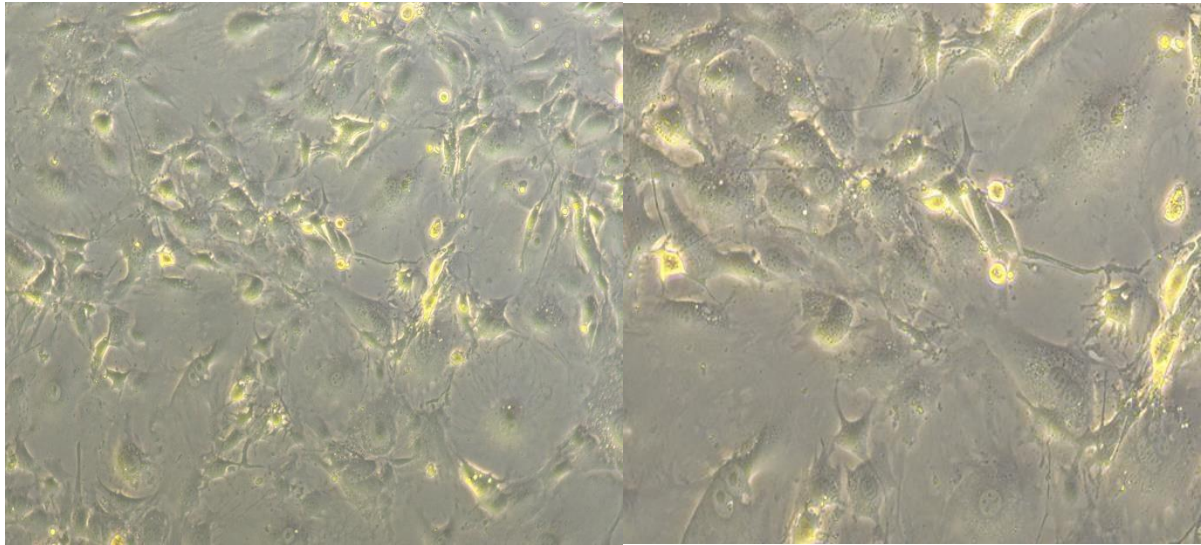
E) Day 8 in differentiation (10X and 20X)



F) Day 10 in differentiation (10X and 20X)



G) Day 12 in differentiation (10X and 20X)



H) Day14 in differentiation (10X and 20X)

Figure 12. A-B-C-D-E-F-G-H) Differentiation of 3T3-L1 cells under inverted microscope (10X and 20X).

3.8. The evaluation of mature adipocytes the by Oil Red Staining

A simple, reliable, and well-used method of analyzing adipocyte differentiation is to determine the amount of lipid accumulation using the lipid-specific stain Oil Red-O. Oil-RedO is specific for triglycerides, the degree of staining is proportional to the amount of cell differentiation, and the dye can be extracted to allow an accurate quantification of differentiation by spectrometry. As Oil-Red-O staining is specific for triglycerides, it is possible to accurately analyze the degree of lipid accumulation within a culture (Dragunow, 2007).

Lipid (Oil Red O) Staining Kit (MAK194-1KT, Sigma-Aldrich, Germany) was used to verify differentiation and evaluate the ability to accumulate triglycerides in 3T3-L1 cells transformed into mature adipocytes (Akinrinde et al., 2018; Hu et al., 2018).

In our study, 60 mg Oil Red-O was diluted with 20 mL 100% Gsopropanol, it was mixed well and it was waited for 20 minutes. Oil Red O stock solution was mixed with pure water in a ratio of 3:2, was waited for 10 minutes untouched. After waiting time, it was filtered and taken to falcon tube. The solution should be ready 15 minutes before use. It could remain stable for up to 2 hours. 6-well plates were aspirated media. Cells were washed 2 times with

PBS. 2 mL 10% formalin was added to fix the cells. Formalin was added directly to the wall, not to the cells. It was left incubated for about 1 hour. Formalin was excreted and the cells were washed twice with ultra-pure water. 60% Isopropanol amount was added to be 2 mL in each well and the cells were incubated for 5 minutes. Isopropanol was aspirated and Oil-Red O was added to the working solution. The plate was rotated and distributed all over and was waited 10-20 minutes for incubation. Wash the cells with ultra-pure water until the dye comes out. 2 ml of Hematoxyline was added to each well and 1 minute was expected for incubation. By taking Hematoxyline 3-4 times the cells were washed with pure water. The last cells were coated with water and the images were taken under a microscope (Ramirez-Zacarias et al., 1992; Akinrinde et al., 2018; Hu et al., 2018).

3.9. Cytotoxicity

In-vitro studies for cytotoxicity experiments, 0.45 g of plant extracts weighed 10% DMSO (Sigma Aldrich, USA) and 90% medium as stock solution 45 mg/mL, all extracts prepared for the experimental studies and 4 °C was stored at. From plant extract shown high efficiency on 3T3-L1 adipocyte cells in in-vitro studies, stock solution will also be prepared as different concentrations.

3.9.1. MTT assay

MTT is a method of determining the presence of living cells based on color change by taking the living cell and reducing it by mitochondrial enzymes (Van de Loosdrecht et al., 1994). One well-known and simple cell viability test is the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrasodium bromide] assay, a colorimetric assay in which active mitochondria cleave the pale-yellow substrate to yield a dark-purple formazan product (Chemicon International, Temecula, CA). Therefore, in our project, MTT assay (Sigma-Aldrich, Germany) was used to evaluate the effect of *B.spergulifolius* extracts on 3T3-L1 adipocyte cell viability. After the cells have covered the flask base by 85-90%, the remaining cells in the flask base was counted by staining with trypan blue, 3T3-L1 adipocyte cells (2×10^3 cells/well) was cultured on 96-well plate and incubated overnight at 37 °C under 5% CO₂ (Freshney, 2000; Akinrinde et al., 2018; Hu et al., 2018). After the incubation of 3T3-L1 adipocyte cells, When the cells reached at 85-90% density and were treated with different concentrations was applied to the cells and incubated for 48 hours (Shin et al., 2013).

For MeOH extract; 0-10-25-50-100-150-200-250-300-350-400-450-500-1000 µg/mL,

For EA extract; 0-10-25-50-100-150-200-250-500-1000-1250-1500-1750-2000 µg/mL,

For Aqueous extract; 0-10-25-50-100-150-200-250-500-550-600-650-700-750-1000 µg/mL,

Experiments were replicated three times and data evaluated with standard deviations. As a control group, DMEM (Hyclone, GE Healthcare, USA) was added as sterile DMSO as the highest concentration of plant extract instead of test substances. The medium was aspirated from 96-well plates (Figure 13). The cell surface was washed 2 times with PBS. In the adipocyte medium in the Falcon tube so that MTT in powder form is 0.5 mg/m µL are solved.

MTT was added to each well to be 100 µL. Cells are incubated at 37°C for 3-4 hours. The medium is aspirated after incubation. 100 µL DMSO is added to each well. 96-well plates wrapped with aluminum foil and for about 1 hour put in the shaker. Measurements are made 3 times for each sample in the nanodropa after thoroughly mixing. These measurements were made at 570 nm. With the results obtained by MTT method, inhibition concentration of adipocyte cells i.e. concentration of plant extract inhibited by proliferation of 50% of adipocyte cells (IC₅₀) was calculated and the anti-diabetic effects of plant extracts was investigated in further research. After determination of the effective dose of each plant extract, EA (Ethyl acetate extract applied), MeOH (Methanol extract applied to), Aqueous (applied Aqueous extract), Control (not any application has been made) and Control+DMSO (DMSO applied) further research was carried out by creating experimental groups (Özdağ and Özdağ, 2018; Özdağ, 2019). The percentage of viability was calculated with following formulation:

Cell viability (%) = (Avg. OD₅₇₀ of treated cells) / (Avg. OD₅₇₀ of control) x 100.

Avg: Average

OD₅₇₀: Optical density

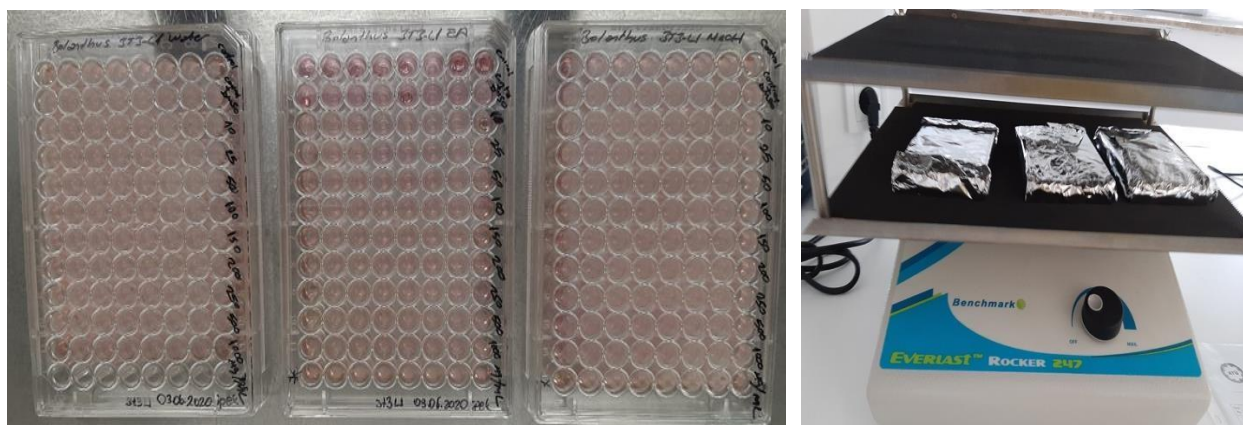


Figure 13. A-B-C) 96-well plates for MTT assay

3.10. Scratch assay

Scratch assay was performed to determine the effect of *B. spergulifolius* extracts on the cell migration ability of 3T3-L1 adipocytes (Hulkower and Herber, 2011). The 3T3-L1 adipocytes were seeded into 6-well plate (3×10^5 cells/well). When the cells reached approximately confluence, the scratch-wound in the middle of each well was created with a 200 μ L sterile micropipette tip. Then wells were removed and washed with DPBS. Medium having IC_{50} dosage of *B. spergulifolius* extracts were added to each well. Preadipocytes and adipocytes as controls received respectively, only fresh medium and adipocyte maintenance medium. All cells were incubated under standard conditions. The images of the scratchwound were taken at 0. h, 6. h, 24. h, 30. h, 48. h using microscope (4X magnification). Also, the scratch-wound area was measured from the digital images and calculated using ImageJ software (imagej.nih.gov) (Valid, et al 2020). The scratch closure rate (%) were calculated:

The scratch closure rate (%) = $\frac{[(\text{Initial scratch-wound width} - \text{Final scratch-wound width}) / (\text{Initial scratch-wound width})] \times 100}{1}$.

3.11. qRT-PCR

To investigate effect of *B. spergulifolius* on apoptosis and insulin resistance, respectively the expression of *Bcl-2-associated X (Bax)*, *B-cell lymphoma-2 (Bcl-2)* and *Glucose transporter-4 (Glut-4)* genes at mRNA level in the 3T3-L1 adipocytes following treatment of extracts was determined using quantitative real-time polymerase chain reaction (qRT-PCR) (Porter and

Jänicke, 1999; Singh and Kakkar, 2013). The 3T3-L1 adipocytes were seeded into 6-well plate at an initial density of 3×10^5 cells/well. The total RNA was isolated from the cells using TRIzol reagent 24 h of treatment with plant extracts according to the product manufacturer's protocol. The RNA was dissolved in DEPC water and stored at -80 °C until it was used. The concentration of RNA samples was measured using a spectrophotometer (Nanoprop 2000c, Thermo Scientific, USA). The 1 µg of the total RNA with 260/280 nm ratio: 1.8-2.1 were used for complementary DNA (cDNA) synthesis. ProtoScript II first Strand cDNA Synthesis Kit (NEB, New England Biolabs Inc., USA) was used to convert the RNA to cDNA following instructions of the manufacturer. The amplification of cDNA was prepared using the One Taq 2X Master Mix with Standard Buffer (New England Bio labs Inc., USA) in a final volume of 25 µL using thermal cycler (SureCycler 8800, Agilent Technologies, USA) with using primers synthesized by Sentebiolab, Turkey (Table 4). The qRT-PCR was carried out using the GoTaq® 2-Step RT-qPCR System (Promega, ABD) on the Qiagen Rotor-Geneq (Qiagen, Hilden, Germany) by following PCR steps summarized in table 4. The expression of target genes was analysed with the comparative threshold cycle CT method ($\Delta\Delta CT$) method, by which the results of each gene expression were normalized with expression of *Gapdh* gene (Livak and Schmittgen, 2001). The results of qRT-PCR were analysed with Rotor Gene Q Software 1.2 program (Qiagen, Germany). All PCR products performed at the same time was displayed on the gel with agarose gel electrophoresis, checking whether primers replicate the correct target region and with negative controls (Özdağ and Özdağ, 2018; Özdağ, 2019).

Table 4. Genes and sets of the primers used in qRT-PCR

Gene	Gene Bank Accession ID	Primer forward 5'-3' nucleotide sequences	Primer reverse 5'-3' nucleotide sequences	Product length (bp)
<i>Bax</i>	NM_007527.3	CCAGGATGCGTCCACCAAGA	GGTGAGGACTCCAGCCACAA	443
<i>Bcl-2</i>	NM_009741.5	TGAGTACCTGAACCGGCATCT	GCATCCCAGCCTCCGTTAT	57
<i>Glut-4</i>	NM_001359114.1	CTTCTTTGAGATTGG CCCTGG	AGGTGAAGATGAAGAAGCCAAGC	215
<i>Gapdh</i>	NM_001289726.1	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	690

Bax: Bcl-2-associated X; *Bcl-2*: B-cell lymphoma 2; *Glut-4*: Glucose transporter 4; *Gapdh*: Glyceraldehyde-3phosphate dehydrogenase

3.11.1. Isolation of RNA from monolayer cell culture

To investigate effect of *B. spregulifolius* on apoptosis and insulin resistance, respectively the expression of *Bcl-2-associated X (Bax)*, *B-cell lymphoma-2 (Bcl-2)* and *Glucose transporter-4 (Glut-4)* genes at mRNA level in the 3T3-L1 adipocytes treatment of extracts was determined using quantitative real-time polymerase chain reaction (qRT-PCR) (Porter and Jänicke, 1999; Zhu, et. al., 2015; Lee, et. al., 2017). For this reason, in this study, total RNA isolation was performed primarily.

Firstly, 3T3-L1 cells were planted in 6-well plates in 8×10^4 numbers for each well. At the end of 24-hour incubation of 3T3-L1 mature adipocyte cells, 85-90% density is obtained by removing the media on the cells, IC_{50} doses of *B. spregulifolius* extracts were administered to cells. IC_{50} doses of *B. spregulifolius* extracts were administered in the form of 3 repetitions for each sample. After 48 hours the application of *B. spregulifolius* extracts, the process of RNA isolation began. 400 μ L of TRIzol (93289 TRI Reagent, Sigma-Aldrich, Germany) was added in the fume hood. Pipetage was performed 10-15 times to lift and homogenize the cells, then the entire surface was scraped with the tip of the pipette. All lysates were taken into separate 1.5 mL ependorf tubes for each well. Incubated for 5 minutes at room temperature. 80ml of chloroform was added and swayed for 2-3 minutes until the color changed. It was centrifuged at 4 °C for 15 min at 12.000 rpm. The transparent part at the top was transferred to the new 1.6 mL ependorf tubes. 200 μ L 2-propanol was added. It was shaken and stirred and incubated at room temperature for 10 minutes. It was centrifuged at 4 °C for 10 min at 12.000 rpm. It was carefully discarded without damaging supernatant RNA. 400 μ L 75% ethanol was added. Stirred with a slight shake. It was centrifuged at 5 min 4 °C at 12.000 rpm. The supernatant was discarded and was left at room temperature for 10 minutes to remove the alcohol. By adding 20 μ L DEPC Treated Water, the RNA was dissolved by pipette (Figure 14). Measurements of RNAs were made at 260 nm in the spectrophotometer.



Figure 14. Total RNA isolation from 3T3-L1 adipocyte

3.11.2. Measurement of the amount of isolated total RNA

Spectrophotometer for concentration measurements of total RNAs isolated from cells Nanoprop 2000c (Thermo Scientific, USA) was used. RNAs ice during measurement it was kept inside. Used to decode RNA in the device'S RNA mode for measurements DEPC

Treated Water was read as "Blank". Solved for nucleic acid concentration Absorbance (Abs) at wavelengths of 230 nm, 260 nm and 280 nm, using 2 μ L from RNA) measured (Figure 15). Quality standard Abs_{260} / Abs_{280} ratio 1.8, purity as a standard, Abs_{260} / Abs_{230} ratio was higher than RNA the concentration of Abs was measured at 260 nm and the value was given as ng/ μ L. Isolated measurement completed total RNAs were maintained at -80°C (Nauire, UK).

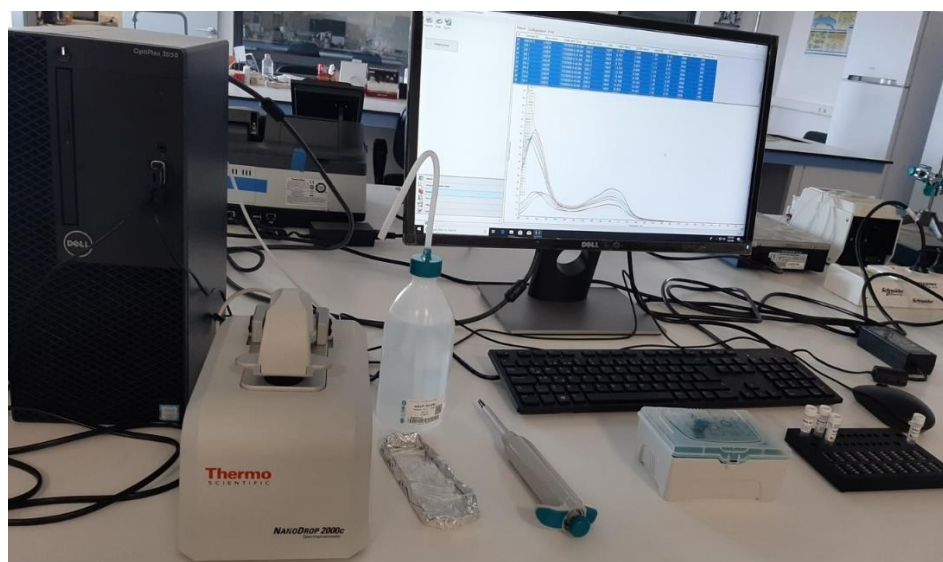


Figure 15. Measurement of isolated total RNA by spectrophotometer

3.11.3. cDNA Synthesis

Isolated RNAs are complementary DNA (cDNA) for use in amplification studies, translated into the form. For translation of RNAs into cDNA form ProtoScript II first Strand cDNA Synthesis Kit (NEB #E6300S/L, New England Biolabs Inc., USA) was used (Figure 24). cDNA according to the results of the measurement before starting the study all RNA samples to be translated into its form were synchronized to 1 µg. First of all, the kit contents the ingredients dissolved on ice. The dissolved reagents were given short-term spin. Firstly, a sterile RNase-free tube consisting of the components in Table 5 was prepared. The mixture in Table 6 was prepared on ice in a separate tube for each RNA sample. Later tubes incubated 5min at 70 °C in surecycler 8800 Thermal Cycler (Agilent, USA) device it was denatured (Figure 16). This process is for long mRNAs and GC-rich RNA regions increases cDNA yield. After incubation, the tubes are given short-term spin, again it was taken on ice. After this stage in a separate sterile RNase-free tube in table 6 the reagents were prepared in opposite quantities for each sample. Negative control also it was also prepared for the tube in opposite quantities from the reagents in Table 7.

Table 5. Quantities of RNA samples and cDNA primers

Reagent	Quantity (µL)
M-MuLV Reaction Mix (2X)	10
M-MuLV Enzyme Mix (10X)	2
Toplam Volume	12

Table 6. cDNA enzyme mixture components and quantities

Reagent	Quantity (µL)
RNA sample	1 µg (1–6)
Anchored oligo d(T)23 VN Primer (50 µM)	2
Nuclease-free H ₂ O	The total amount is set to be 8
Toplam Volüm	8

Table 7. Components and quantities for negative control

Reagent	Quantity (µL)
M-MuLV Reaction Mix	10

H ₂ O	2
Total Volume	12

The mixture prepared from the components given in Table 5 and 6 is mixed with pipette, the first 8 prepared the μ L RNA-primary mixture was distributed to the tubes with each tube to be 12 μ L. This at the end of the process, a total volume of 20 μ L of RNA-enzyme mixture was formed in each tube. Negative tubes with control to surecyclcr 8800 Thermal Cycler (Agilent, USA) was placed. 5 minutes at 25 °C, 60 min at 42°C, and 5 at 80 °C for enzymeinactivation, was incubated. At the end of the process, the samples were taken from the device and kept at - 20 °C.

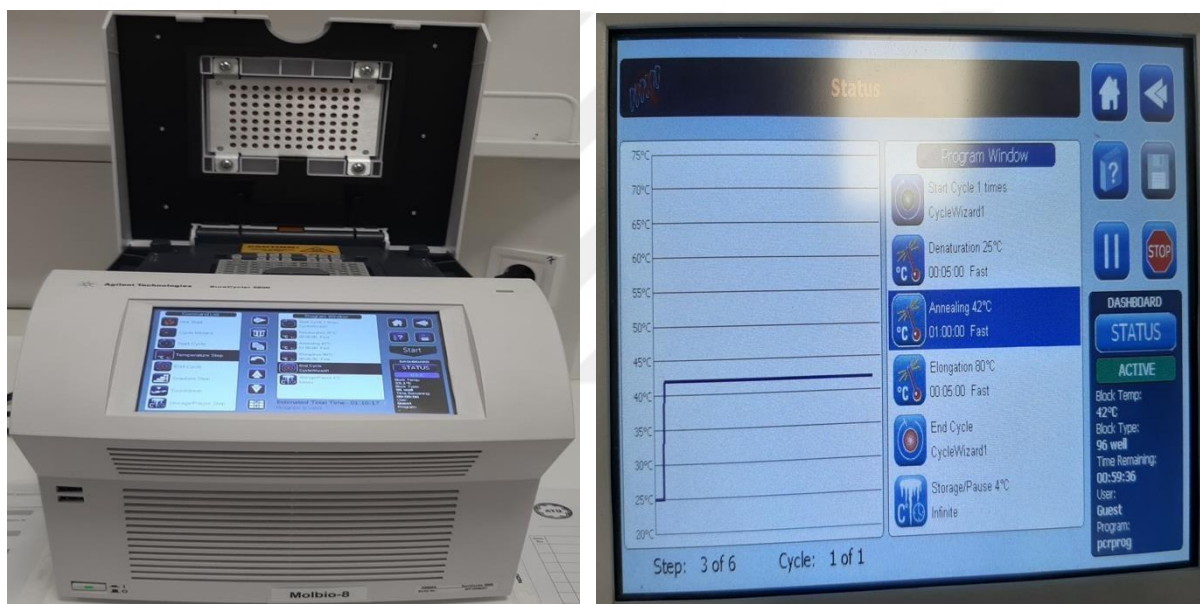


Figure 16. Thermal cycler

3.11.4. PCR and primer design for qRT-PCR

When designing primers, each primer should be 20 bases long, the probability of self-folding should be minimal, and primer pairs should be unlikely to be complementary to each other. Therefore, these parameters were taken into consideration when designing the primer in our study. Since gene expression at mRNA level will be evaluated by PCR and qRT-PCR in our study, primers including 2 exons were chosen when selecting primers. On the National Center for Biotechnology (NCBI) home page, the target gene was named “*Glut-4*” [Glut4 solute carrier family 2 (facilitated glucose transporter), member 4)] by selecting the “Gene” tab and the cell line where the target gene will be investigated is called “*Mus Glut-4* (Slc2a4; Gene ID: 20528)

was selected from here because it is a "*Mus musculus*" organism (Figure 17) (<https://www.ncbi.nlm.nih.gov>). Specific mRNA primers for target gene amplification checked with Primer-BLAST interface (Ahn vd., 2006; Singh ve Kakkar, 2013).

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 Items: 1 to 20 of 36 Selected: 1
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Name/Gene ID	Description	Location	Aliases	MIM
Slc2a4 ID: 25139	solute carrier family 2 member 4 (<i>Rattus norvegicus</i> (Norway rat))	Chromosome 10, NC_005109.4 (66559921..66560562, complement)	Glut4	
SLC2A4 ID: 6517	solute carrier family 2 member 4 (<i>Homo sapiens</i> (human))	Chromosome 17, NC_000017.11 (7281718..7289267)	GLUT4	138190
Slc2a4 ID: 20526	solute carrier family 2 (facilitated glucose transporter), member 4 (<i>Ovis aries</i> (sheep))	Chromosome 11, NC_000077.7 (69833112..69839040, complement)	GT2, Glut, Glut-, Glut-4, Glut4, lwyg	
SLC2A4 ID: 403673	solute carrier family 2 member 4 (<i>Canis lupus familiaris</i> (dog))	Chromosome 5, NC_006887.3 (32238095..32244022)	GLUT4	

Genomic regions, transcripts, and products

Genomic Sequence: NC_000077.7 Chromosome 11 Reference GRCh38 C57BL/6J

Go to reference sequence details

Go to nucleotide: Graphics FASTA GenBank

Genes, NCBI Mus musculus Annotation Release 109, 2020_03

Biological regions, aggregate, NCBI Mus musculus...
 RNA-seq exon coverage, aggregate (filtered), NCBI Mus musculus Annotation Release 109 - log base 2 scaled
 RNA-seq intron-splicing reads, aggregate (filtered), NCBI Mus musculus Annotation Release 109 - log base 2 scaled
 RNA-seq intron features, aggregate (filtered), NCBI M...

NC_000077.7: 70M..70M (7,707 nt) C

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Nucleotide Advanced Search Help

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GenBank =

PREDICTED: Mus musculus solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4), transcript variant X3, mRNA

NCBI Reference Sequence: XM_006532655.4
 FASTA Graphics

Go to: (2)

LOCUS DEFINITION XM_006532655 2301 bp mRNA linear ROD 21-SEP-2020
 PREDICTED: Mus musculus solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4), transcript variant X3, mRNA.

ACCESSION VERSION XM_006532655.4
 DBLINK BioProject: PRJNA169

KEYWORDS SOURCE RefSeq.
 ORGANISM Mus musculus
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia; Myomorpha; Muridae; Muridae; Musinae; Mus; Mus.

COMMENT MODEL REFSEQ: This record is predicted by automated computational analysis. This record is derived from a genomic sequence (NC_000077.7) annotated using gene prediction method: Gnomon, supported by EST evidence.
 Also see:

Change region shown
 Customize view
 Analyze this sequence
 Run BLAST
 Pick Primers
 Highlight Sequence Features
 Find in this Sequence
 Show in Genome Data Viewer

Articles about the Slc2a4 gene
 Changes in Expression of Follicular Glucose Transporters May Be Inv [Ann Clin Lab Sci. 2019]
 A novel mutation in Slc2a4 as a mouse model of fatigue. [Genes Brain Behav. 2019]
 Insulin-responsive amino peptidase follows the Glut4 pathway but is dispens [Mol Biol Cell. 2019]

Primer-BLAST » JOB ID: JS_TAFAN_aXam-ee6v7DrJDI0p699smDvA

Primer-BLAST Results

Input PCR template: none
 Specificity of primers: Target templates were found in selected database: Refseq mRNA (Organism limited to Mus musculus)
 Other reports: Search Summary

Detailed primer reports

Primer pair 1	Sequence (5'→3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTCTTTGAGATTGGCCCTGG	21	58.90	62.38	4.00	3.00
Reverse primer	AGGTGAAGATGAAGAAGCCAAGC	23	61.12	47.83	2.00	2.00

Products on target templates
 >NM_009204.2 Mus musculus solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4), transcript variant 1, mRNA

product length = 215
 Forward primer 1 CTCTTTGAGATTGGCCCTGG 21
 Template 1388 1408
 Reverse primer 1 AGGTGAAGATGAAGAAGCCAAGC 23
 Template 1602 1580

Figure 17. Primer design

3.11.4.1. Gradient PCR

In order to determine the binding temperatures of primers, a PCR study was conducted primarily for their target genes. In this context, cDNA synthesized from high quality RNA obtained from Control cells was used as an example. The primary sequences were used for amplification of the target gene.

Buffer Master Mix, forward and reverse primer ependorf tube contained in the One Taq 2X Master Mix with Standard Buffer (M0482S, New England Biolabs Inc., USA) kit is placed in the quantities specified in the table (Table 8). To obtain 1 µg of DNA, the amount of DNA to be taken was determined and the cDNA we obtained from the cells previously was used. DNA volume was calculated and completed to 2 µL with DEPC. Since there will be a total of 25 µL of solution in each tube, the remaining amount was completed with DEPC. The whole experiment took place on ice.

Table 8. Components and quantities required for PCR operation

Components	Quantity (μL)
Buffer Master Mix	12.5
20 pmol Forward Primer	0.5
20 pmol Reverse Primer	0.5
cDNA	2
DEPC Water	9.5

Using the SureCycler 8800 (Agilent Technologies, USA) PCR device, the program was set under the conditions specified in the table and the total volume in the program was entered as 25 μL. The reaction was initiated by selecting the “Run” option. After the reaction, the products were lifted to -20 °C. PCR reaction conditions are shown in Table 9.

Table 9. PCR Reaction

Reaction	Temperature (°C)	Time min	Cyle
Hot start	94	3	1
Denaturation	94	1	1
Denaturation	94	1	35
Primary binding	60	1	35
Elongation	72	1	35
Final elongation	72	10	1

3.11.5. Agarose gel electrophoresis

Agarose gel electrophoresis was used to display amplified DNA molecules as a result of the PCR study. 10X Tris boric acid EDTA (TBE) buffer: 10X TBE has been prepared as a buffer solution for use both for the preparation of the gel and in the tank where the gel will be executed in agarose gel electrophoresis.

Ingredients:

- Boric acid
- Tris (Hydroxymethyl) Aminomethane

- Ethylenediaminetetraacetic acid (Na₂EDTA)
- 54 g Tris, 27.5 g of boric acid and 7.44 g of Na₂EDTA were weighed. The chemicals that were weighed were put in a magnetic blender to dissolve in pure water. After the PH is set to 8.3, it was completed to 1 liter with pure water. The buffer solution was stored at room temperature.

To achieve 0.5 X TBE, 50 mL 10X TBE buffer was taken and it was completed with 1 liter of pure water. A total of 60 mL 0.5 x TBE buffer was used for 2 gels. To prepare 2% agarose gel, 1.2 g agarose was weighed. Agarose and buffer solution were slightly shaken and mixed, then the top was covered with aluminum foil. Erlenmeyer was microwaved for about 2 minutes at 600 W. It was waited a little to cool, then 1.5 µL SYBR Safe DNA Gel Stain was added (S33102, Invitrogen, Thermo Fisher Scientific, USA). The mixture was poured into the gel tray and the comb was placed. It was waited 20 minutes for the gel to solidify. After the gel solidified, the combs were removed. The gel was placed in the electrophoresis chamber and a sufficient amount of 0.5X TBE buffer was poured into the electrophoresis chamber. Mixed with 0.5 µL 6X Loading Dye (R0611, ThermoScientific, USA) on Parafilm by taking 5 µL from each DNA. Loaded into the well to be a 1 kb DNA marker in the first well. The electrophoresis tank was connected to the power supply and was executed at 90 V for about 45 minutes.

3.11.6. Display of gels under UV light

After completion of execution, BioSpectrum 810 (UVP, USA) UV transilluminator device was used to display agarose gel under UV. The cover of the device is opened and placed in the gel and the UV light is opened with the UV button and the cover is closed. The resulting images were recorded.

3.11.7. DNA extraction from agarose gel

Nucleospin Gel and PCR Clean-up kit (740609.50, MACHEREY - NAGEL, Germany) was used for DNA extraction from agarose gel. Agarose gel found in a piece of DNA was cut and removed with a clean scalpel. It was placed in gel elendorf, which weighed 260 mg. 520 µL Binding Buffer NTI was added on it. Incubated for 10 minutes at 50 °C in a water bath. Every 3 minutes, the tube was vortexed and the gel dissolved. The purification column contained in the Nucleospin Gel and PCR Clean-up kit was transferred from 700 µL of soluble gel and was placed under the collection tube. It was centrifuged at 11.000 g for 30 seconds. Collection tube

was removed. 700 μL Washing Buffer NT3 was placed in the column. It was placed in microcentrifuge and operated at 11,000 G for 30 seconds. This process was repeated 2 times. It was centrifuged again for 1 minute without adding anything to completely get rid of the NT3 buffer. The colon was taken into a new ependorf tube. A 30 μL Buffer NE was added to the column and left to incubate for 1 minute at room temperature. It was last centrifuged at 11.000 rpm for 1 minute and DNA was obtained in the ependorf tube. Then the “Nuclear Acid” part of the spectrophotometer Nanoprop 2000c (Thermo Scientific, USA) was selected and measured.

Spectrophotometer Nanoprop 2000c (Thermo Scientific, USA) was used for concentration measurements of DNA obtained by extracting agarose gel. For measurement, it was first read to the device as an Eution Buffer blank, in which DNA is thawed in DNA mode. Abs measurements were performed at wavelengths of 260 nm and 280 nm using 2 μL from the dissolved DNA for DNA concentration. DNA that was measured was stored at -20°C (Nauire, UK).

3.11.7.1. Standart preparation for qRT-PCR

Standards for qRT-PCR analyses were prepared using DNA obtained for each extracted target gene. DNA concentrations were determined as *Gapdh*: 12.71 ng/ μL , *Bax*: 10.77 ng/ μL , *Bcl-2*: 8.65 ng/ μL and *Glut-4*: 7.83 ng/ μL , respectively. For standard preparation, these units were primarily translated as “pg”. First standards and serial dilutions were prepared by diluting Nuclease Free Water.

Gapdh: 12.71 ng/ μL = 13000 pg $\rightarrow$ 130 X 100x $\rightarrow$ 1 μL DNA + 129 μL Nuclease Free Water = Standart 1

Bax: 10.77 ng/ μL = 10800 pg $\rightarrow$ 108 X 100x $\rightarrow$ 1 μL DNA + 107 μL Nuclease Free Water = Standart 1

Bcl-2: 8.65 ng/ μL = 8700 pg $\rightarrow$ 87 X 100x $\rightarrow$ 1 μL DNA + 86 μL Nuclease Free Water = Standart 1

Glut-4: 7.83 ng/ μL = 7900 pg $\rightarrow$ 79 X 100x $\rightarrow$ 1 μL DNA + 78 μL Nuclease Free Water = Standart 1

10 μL Standart 1 + 90 μL Nuclease Free Water = Standart 2

10 µL Standart 2 + 90 µL Nuclease Free Water = Standart 3
 10 µL Standart 3 + 90 µL Nuclease Free Water = Standart 4
 10 µL Standart 4 + 90 µL Nuclease Free Water = Standart 5
 10 µL Standart 5 + 90 µL Nuclease Free Water = Standart 6
 10 µL Standart 6 + 90 µL Nuclease Free Water = Standart 7

3.12. Evaluation of *Bcl-2*, *Bax* and *Glut-4* gene expressions in 3T3-L1 adipocyte cells by qRT-PCR

The apoptotic pathway is controlled by regulation of mitochondrial permeability of *Bax/Bcl-2*, and *Bax*, *Bcl-2* gene expression has been shown to be associated with apoptosis due to oxidative stress (Ahn et. al., 2006; Zhu et. al., 2015; Lee v et. al., 2017). Increased *Glut-4* in cell plasma membranes following insulin stimulation is closely associated with increased glucose uptake in adipocytes and skeletal muscle cells. Therefore the IC₅₀ dose of *B. spargulifolius* extracts which are applied in mature adipocytes 3T3-L1 transformed into experimental and control groups applied cells to investigate the effect on apoptosis and insulin resistance, to determine *Bax*, *Bcl-2* and *Glut-4* gene expression, qRT-PCR method was performed. qRT-PCR study was performed with the GoTaq® 2-Step RT-qPCR System (A6110, Promega, USA) on the QIAGEN Rotor-Geneq (Qiagen, Germany) device to determine the efficiency at the level of mRNA with the standards prepared for cDNAs obtained from 3T3-L1 cells. The mastermix was prepared in triplicate for each sample and standard with a final volume of 20 µL, and the qRT-PCR study was performed by setting it for 45 cycles at the appropriate binding temperature for each primer set in Rotor Gene Q (Qiagen, Germany) (Table 10). *Gapdh* was used for normalization of the expression of target genes and the results were analyzed with the comparative threshold cycle CT method; $\Delta\Delta CT$ (Livak and Schmittgen, 2001). The results after qRT-PCR were analyzed in Rotor Gene Q Software 1.2 program (Qiagen, Germany). GoTaq® qPCR Master Mix PCR components are shown in Table 10.

Table 10. GoTaq® qPCR Master Mix PCR components

Components	Quantity (µL)
GoTaq® qPCR Master Mix	10

Forward Primer	0.5
Reverse Primer	0.5
Supplemental CXR Reference Dye	0.2
cDNA (1 µg)	—
Nuclease-Free Water	to a final volume of 20

3.13. Evaluation of Glut-4 protein expressions in 3T3-L1 adipocyte cells by Western-blot

Western blot is often used in research to separate and identify proteins. In this technique, a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest (Mahmood, 2012).

3.13.1. Protein isolation and quantity measurement

EA, MEOH, Aqueous, C-medium to determine the level of the target protein in the cells, the mediums of cells located in 6-well plate were completely removed. A total of 250 µL (90% lysis buffer+10% protease inhibitor) suspension containing 225 µL Radio-immunoprecipitation-assay buffer (RIPA) (R0278, Sigma) and 25 µL Protease Inhibitor Cocktail (P8340, Sigma) was added for each well in the 6-well plate. The suspension was shaken at +4

° C for 15 min. Each well was scraped with a cell scraper, pipetting was done several times. The tubes were placed in the sonicator device (Bioruptor sonicator, Belgium). 10 cycles of high level sonication was performed at 30"/30" (ON/OFF cycles) interval. After that, the tubes were centrifuged for 10 min at 15.000 rpm. The Protein was taken from the supernatant. The proteins were measured in Nanoprop 2000c (Thermo Scientific, USA) device using the Bradford method.

Quick Start Bradford Protein Assay Kit 1 (500-0201, Bio-Rad, Germany) was used to Bradford Method. Quick Start bovine Serum Albumin (BSA) 2 mg/mL (500-0206, Bio-Rad, Germany) in the kit was prepared to be in the range of 2,000 µg – 0 µg as outlined in Table 11 below for measurement. Each 5 µL 250 µL standard and sample taken over from the kit is

located in the Quick Start Bradford 1X Dye Reagent (500-0205, Bio-Rad, Germany) were mixed and incubated at room temperature for about 30 minutes with, and then 2000C of the nanoprop spectrophotometer (Thermo Scientific, USA) “Protein Bradford” application by selecting at a wavelength of 595 nm by reading a standard curve was plotted. Water and Quick Start Bradford 1X Dye Reagent were used as “blank”. The protein concentration was determined in each sample with a standard-based analysis of the measurement results, and for Western-blot studies, the protein concentrations in the samples were equated to 20 µg.

Table 11. Standard preparation for micro-measurement

Tube	Standart volume (µL)	Standard Source	Dilution volume (µL)	Final protein concentration (µg/mL)
1	20	2 mg/mL stock	0	2.000
2	30	2 mg/mL stock	10	1.500
3	20	2 mg/mL stock	20	1.000
4	20	Tube 2	20	750
5	20	Tube 3	20	500
6	20	Tube 5	20	250
7	20	Tube 6	20	125
8 (blank)	-	-	20	0

3.13.2. Western-blot

After measurement, the proteins were mixed with the sample buffer containing 5% Mercaptoethanol (Sigma-Aldrich, Germany). Western-blot system in our laboratory has been optimized in previous studies, Western blot protocol is used, and proSemi-Dry Blotting (Bio-Rad, USA) system (Özdağ, 2018; Özdağ and Özdağ, 2018; Özdağ, 2019). Protein analysis was carried out using devices and expenses belonging to this system. The proteins were equalized to 20mg and loaded into 10% Sodium-Dodesylsulfate (SDS-PAGE) gel in appropriate quantities and separated according to their size by executing 100 V 5 min and 240 V 17 min in the ProSieve™ EX Running buffer (Lonza Bioscience, USA). The Blotter (Bio-Rad, USA) device was used to transfer proteins in the gel to the PVDF membrane. After the Transfer process is completed, the membranes were blocked in 5% skimmed milk powder first after 1-2 minutes of waiting in 100% methanol.

After blocking, the membranes were left overnight on the shaker at +4 °C with anti-*Glut-4* (1:100) as the first antibody and anti- β -actin-antibody (1:100) as a positive control. At the end of the period, the first antibodies were taken into the Falcons and the preservative was added and removed to the refrigerator at +4 °C for later use. Then the membranes were washed three times with 1X TBST. After washing, it was incubated on Shaker for 1 hour at room temperature with goat Anti-mouse IgG HRP-linked secondary antibody (HRP) (1:1000) for *Glut-4* and β -actin as secondary antibody in 1X TBST. At the end of incubation, the membranes were washed three times with 1X TBST. After washing, the membranes were incubated in 2-3 min darkness with a 1:1 solution of ECL (Bio-Rad, #170-5060). Finally membranes for displaying VersaDoc (4000MP, Bio-Rad, USA) device:

On the device of VersaDoc (4000MP, Bio-Rad, USA), these buttons were clicked respectively; New single channel > Blots Ch. > High Sensitivity > Select gel type > Mini protean > Position gel. Then, the image of the gel was displayed in the middle of the screen by clicking the "Run protocol" button (Figure 18).



Figure 18. Western-Blot VersaDoc (4000MP, Bio-Rad, USA) imaging device

3.14. Immunofluorescence evaluation of intracellular level of *Glut-4* proteins in 3T3-L1 adipocyte cells

To investigate the effect of determined effective doses of *B. spargulifolius* extracts on oxidative stress-related apoptosis and inert resistance in 3T3-L1 cells transformed into mature adipocytes,

an immunofluorescence staining method was applied to evaluate the intracellular level of Glut-4 protein. For this purpose, the cells were first planted in 3×10^5 numbers on 13 mm nunc Thermanox Coverslips (Thermo Scientific, nunc 174950), which were placed 3 pieces in each well in a 6-well plate. 24 hours after planting, the slips in the 6-well plate were transferred to the 24-well plate with the help of a sterile injector needle and tweezers, so that there was one slip to each well.

250 μ L 4% (w/v) paraformaldehyde (1X PBS dissolved in PH 7.4) was incubated at room temperature for 10 min for fixation on slips transferred to 24-well plate. Then paraformaldehyde was pulled from the cells and the slips were washed 3 times with cold 300 μ L 1X PBS. Permeability is very important because the target protein is intracellular. After washing, 250 μ L of 0.1% Triton X-100 (dissolved in 1X PBS, v/v) was incubated at room temperature for 10 minutes on the slips for permeabilization. Triton X-100 is the most common detergent used to improve antibody penetration. The Triton on the cells was pulled and washed in 250 μ L 1X PBS three times for 5 minutes. To prevent nonspecific binding of antibodies after permeabilization, their cells were blocked by adding 250 μ L 1% (w/v) BSA [thawed in PBST (PBS + 0.1% Tween 20)] and incubated for 30 minutes at room temperature. Blocked cells were incubated with 1:100 (v/v) of anti-Glut4 (Anti-Glucose

Transporter Glut-4 antibody [1F8] ab35826, Abcam, Cambridge, UK) in 250 μ L 1% (w/v) of BSA in [PBST (PBS + 0.1% Tween 20)] and 250 μ L 1:100 (v/v) of anti-Beta Actin in 250 μ L 1% (w/v) of BSA in [PBST (PBS + 0.1% Tween 20)] for overnight at 4 °C in a humidified room.

For the negative control, only 250 μ L of 1% BSA (w/v) (dissolved in PBST) was used and incubated overnight (or 1 hour) at +4 °C without applying the first antibody. At the end of the incubation, the antibody solution was removed and the cells were washed three times in 250 μ L 1X PBST for 5 minutes. After washing all wells; For Glut-4 and Beta-actin, Goat Antimouse IgG H&L (Alexa Fluor® 488) was added 1/1000 in 250 μ L 1% BSA (w/v) (dissolved in PBST) and it was incubated at room temperature in the dark for 60 minutes. At the end of the incubation, the second antibody solution was removed and the cells were washed twice in 250 μ L 1X PBST for 5 minutes in the dark.

Afterwards, DAPI (1 µg/mL) (DNA dye) was prepared at the rate of 1/1000 (v/v) in 250 µl 1X PBS for nuclear staining and added to each well and incubated for 5 minutes. At the end of the incubation, DAPI was removed and cells were washed twice for 5 minutes in 250 µL of 0.1% BSA (dissolved in PBST). Finally, 300 µL 1X PBS was added to the wells.

The slips, whose staining process was completed, were transferred to slides for visualization. For this, slips were placed on the slide with the cellular part on top. 3 µL BrightMount/Plus was added as a preservative on each slip. After the preservative had dried, slips were viewed under an immunofluorescent microscope stored at -20 °C or +4 °C in the dark. The fluorescent signal the fluorescent microscope (Leica Dmi1 Led Flo) was analyzed using (Liu et al., 2015; Özdağ, 2018; Özdağ, 2019). Staining results are expected to confirm mRNA and protein expression data of target genes. Intra-cell localization, distribution and quantities of target proteins by staining them with specific antibodies were examined.

3.15. Evaluation of the effect of *B. spregulifolius* extracts on 3T3-L1 adipocyte cell viability by fluorescent staining

The effect of IC₅₀ doses of *B. spregulifolius* extracts on cell viability in 3T3-L1 cells transformed into mature adipocytes was evaluated by fluorescent staining. While Eth-1 cannot pass through the membrane of the living cell, it passes through the cell membrane with disrupted integrity and allows the red fluorescence of dead cells by binding to DNA. CalseinAM is a non-fluorescent Acetomethoxy derivative compound that passes through the membrane of living cells, is broken down by Esterase enzymes in the cytoplasm and transforms into Calcein, which fluoresces, making living cells appear green Therefore, chemicals Calsein-AM and Eth-1 were used to identify live/dead cells by immunofluorescent staining.

For this, firstly, the cells were planted in 3X10⁵ numbers on 13 mm Nunc Thermanox Coverslips (Thermo Scientific, Nunc 174950) placed 2 in each well in a 6-well plate. 24 hours after planting, the slips in the 6-well plate were transferred to the 24-well plate with the help of a sterile injector needle and tweezers, so that there was one slip to each well. Fluorescence staining was performed 24 hours after IC₅₀ doses of *B. spregulifolius* extracts were administered to cells with a density of 85-90% at the end of incubation for the evaluation of Living / Dead Cells staining. At the end of incubation, the fattening medium in the cells was removed and the

cells were washed 2 times with 250 μ L Ca⁺⁺, Mg⁺⁺PBS (PBS+) for 24well plates. Then 250 μ L PBS+ cells containing 1 μ M Eth-1 and 1 μ M Calsein-am were added to 24-well plates. The cells were incubated in darkness at room temperature for 30 minutes. At the end of incubation, the staining solution was removed from the environment and washed 1 time with 250 μ L PBS+ and examined under a fluorescent microscope.

3.16. Evaluation of the effect of *B. spregulifolius* extracts on 3T3-L1 adipocyte cell morphology

3.16.1. Staining of cells with Giemsa

To investigate the effect of IC₅₀ doses of *B. spregulifolius* extracts on cell morphology in 3T3L1 cells converted to mature adipocytes, the cells were evaluated by Giemsa staining. At the end of incubation for Giemsa staining, the cells were stained 24 hours after applying IC₅₀ doses determined by MTT of *B. spregulifolius* extracts to cells with a density of 85-90%. So the cells were first planted in 3×10^5 numbers in a 6-well plate. After 24 hours planting, the fattening medium on the cells was removed for fixation. Washing was done 2 times with 750 μ L PBS for 6-well plate. 750 μ L 40% PBS+60% (v/v) methanol mixture was incubated for 1 min and removed. Then, 750 μ L of 100% (v/v) methanol was added and incubated for 10 minutes and fixation process was completed by washing twice with 750 μ L PBS.

0.1 g of Giemsa paint was dissolved in 10 mL of methanol, left in hot water for 20-30 minutes, passed through a filter of 0.42 μ m. 1 mL of Giemsa was incubated at room temperature for 20 minutes by adding a staining solution to the washed cells that had been fixed. After incubation, the dye solution was removed and the cell morphologies were examined under an invert microscope.

3.16.2. Imaging of cells with SEM

In order to investigate the effect of IC₅₀ doses of *B. spregulifolius* extracts on cell morphology in 3T3-L1 cells converted to mature adipocytes, the cells were evaluated with SEM. At the end of incubation for SEM imaging, the cells were displayed with SEM 24 hours after applying IC₅₀ doses of *B. spregulifolius* extracts to cells with a density of 85-90%. For this purpose, the cells were first planted in 3×10^5 numbers on 13 mm nunc Thermanox Coverslips (Thermo Scientific, Nunc 174950), which were placed 2 pieces in each well in a 6-well plate.

After 24 hours transplantation, the coverslip in the 6-well plate was taken with the help of sterile forceps and a clean 12-well plate was taken. For SEM reviews, the cells were fixed for 45 minutes with 2.5% Gluteraldehyde (1010, Agar Scientific, Essex, England) prepared in PBS (pH 7.2). After the detection process, samples were washed 2 times with 1 mL of PBS in 12-well plate to remove fixative and other impurities. After washing, it was dehydrated by passing from the graded 1 mL alcohol series from low to high concentration (30%, 50%, 70%, 80%, 90% and 96%) for 15 minutes. Washed with hexamethylmelamine (HMDS) for 5 min and dried in air for 30 min. Samples were viewed in a thin layer with gold ions by scanning electron microscopy (Carl Zeiss, Supra 40 VP, Germany). SEM photos were examined at 500X magnification.

3.16.3. Actin-filaments staining by fluorescence of the cell nucleus

Actin-filaments and cell nucleus were evaluated by fluorescence staining to investigate the effect of IC₅₀ doses of *B. spregulifolius* extracts on cell-skeleton and morphology in 3T3-L1 cells converted to mature adipocytes. At the end of incubation for F-actin and cell nucleus Florentine staining, fluorescent staining was performed 24 hours after MTT-determined IC₅₀ doses of *Spregulifolius* extracts were administered to cells with a density of 85-90%.

The cells in 24-well plates washed two times with phosphate buffered saline (PBS) and were fixed with 250 µL %4 (w/v) Paraformaldehyde and permeabilised 250 µL %0.1'lik Triton X1000. Then, by blocking with BSA (PBST [PBST (PBS+%0.1 Tween 20) içinde çözdürülmüG], non-selective binding was prevented. Then, 250 µL Phalloidin-iFluor 488 Reagent 1:100 (v/v) in %1 (w/v) BSA and DAPI 1:1000 (v/v). The fluorescent signal was analyzed using the lazer confocal microscope (Leica Dmil Led Flo).

3.16.4. Statistical analysis

Statistical Package for Social Science (SPSS) v25.0 version for statistical analysis of data (Inch., Chicago, IL, USA) package program will be used. All measurements made in the study are three it will be repeated once. The data will be expressed as mean $\pm$ SD and graphs with Excel program will be prepared (Microsoft Office 2013). The data we will obtain in our experiments is normal. Mann-Whitney U in comparison of groups to be checked if they are dispersed or

Student's t-test will be used. Data between more than two independent groups for comparison, Kruskal-Wallis or One-ANOVA test will be used. Anova Tamhane or Bonferroni correction will be used for post-hoc testing. IC₅₀ values, data was analyzed by Graphpad Prism (Graphpad Software Inc.) using the software, to the concentration of the tested sample using the non-linear regression analysis method of the counter-cell inhibition percentage graph were calculated. Statistically value of $P < 0.05$ will be considered significant.



4. RESULTS

4.1. TPCs of *B. spergulifolius*'s extracts

The extraction yield and TPC of *B. spergulifolius* crude extracts were summarized in Table 12. The extraction efficiency of *B. spergulifolius* Aqueous extract ($49.3 \pm 1.05\%$) was the highest, followed by the extraction efficiency of MeOH extract and EA extract ($46.91 \pm 1.60\%$, $23.2 \pm 1.88\%$, respectively) lower.

TPC chelate with metal ions that have the ability to destroy free radicals and form lipid peroxidation and contribute to antioxidant activity by scavenging free radicals (Shi et al., 2005; Bravo, 1998). In our study, TPC of *B. spergulifolius* extracts was evaluated as an indicator of anti-oxidant activity. TPC in *B. spergulifolius* extracts was calculated with FolinCiocalteu reagent and gallic acid equivalent to standard curve equation ($R^2 = 0.9970$). The TPC was highest in the *B. spergulifolius* EA extract (52.81 ± 1.57 mg/g dry weight). Subsequently, the total phenolic content was lower in *B. spergulifolius* MeOH extract (48.22 ± 1.98 mg/g dry weight) and the lowest in *B. spergulifolius* Aqueous extract (30.34 ± 1.36 mg/g dry weight) (Table 12). Each experiment was performed at least 3 times and data were given as mean $\pm$ SD. When the extracts were compared with each other in terms of TPC content, there was a statistically significant difference ($P < 0.05$).

Table 12. Yield of extraction (%) and TPCs of *B. spergulifolius* extracts from different solvents

Extract of <i>B. spergulifolius</i>	Yield of extraction (%, w/w, dry weight)	Total phenolic content (mg GAE/g dry weight)
MeOH	46.91 ± 1.60	* 48.22 ± 1.98
EA	23.2 ± 1.88	* 52.81 ± 1.57
Aqueous	49.3 ± 1.05	30.34 ± 1.36

TPC: Total phenolic content; GAE, gallic acid equivalents; MeOH: Methanol; EA: Ethylacetate; Aq: Aqueous. Yield of extraction expressed as % (w/w). TPC expressed as GAE mg/g extract. Data presented as mean $\pm$ S.D. of three independent measurements for each extract. Statistical analysis was conducted using One-way ANOVA test. * $P < 0.05$, statistically different from Aqueous.

4.2. Cytotoxic effect of *B. spergulifolius* extracts on 3T3-L1 adipocytes

It is a critical step in evaluating the possible cytotoxicity of plant extracts and their suitability for further applications (Mosmann, 1983; Hu et al., 2018). The cytotoxic effect of *B. spergulifolius* extracts of MeOH (0-1000 µg/mL), EA (0-2000 µg/mL) and Aqueous (0-1000 µg/mL) on mature 3T3-L1 adipocyte cell line was investigated with using MTT assay. 96well plates for MTT assay is shown in Figure 33, microscope images of 3T3-L1 adipocyte cells by MTT stained (10X, 20X and 40X, respectively) are shown in Figure 19. After 24 hours of incubation with *B. spergulifolius* extracts, the cell viability results were presented in Figure 33.

Cell viability decreased as the extract concentration increased in all extracts. Cell viability began to decrease significantly at concentrations of *B. spergulifolius* MeOH extract higher than 200 µg/mL compared to control ($P= 0.033$ for 200 µg/mL and $P< 0.001$ for higher doses). Cell viability began to decrease significantly at concentrations of *B. spergulifolius* EA extract higher than 500 µg/mL compared to control ($P< 0.001$ for 500 µg/mL and higher doses). Cell viability started to decrease significantly at concentrations of *B. spergulifolius* Aqueous extract higher than 100 µg/mL compared to control ($P= 0.001$ for 100 µg/mL and $P< 0.001$ for higher doses) (Figure 20A). However, over 90% cell viability was preserved in cells exposed to concentrations of less than 10 µg/mL, 350 µg/mL and 25 µg/mL for *B. spergulifolius* MeOH, EA and Aqueous extract, respectively. Also, final concentrations of 0.1% of DMSO, 0.1% of MeOH and 0.1% EA were not cytotoxic to 3T3-L1 cells.

The extract concentration (IC_{50}) that causes 50% decrease in the viability of the cells was observed as 305.7 ± 5.583 µg/mL, 567.4 ± 3.008 µg/mL and 418.3 ± 4.390 µg/mL for MeOH, EA and Aqueous extract, respectively (Figure 20B, 20C, 20D). Based on this data, the IC_{50} dose of *B. spergulifolius* MeOH, EA and Aqueous extracts was chosen to be applied to the cells in subsequent experiments.

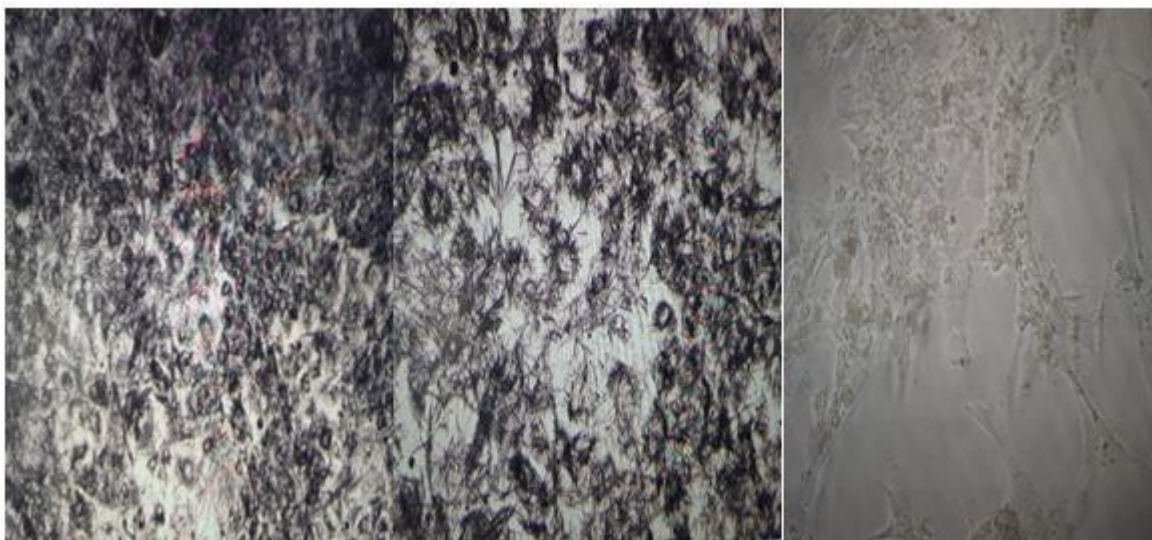
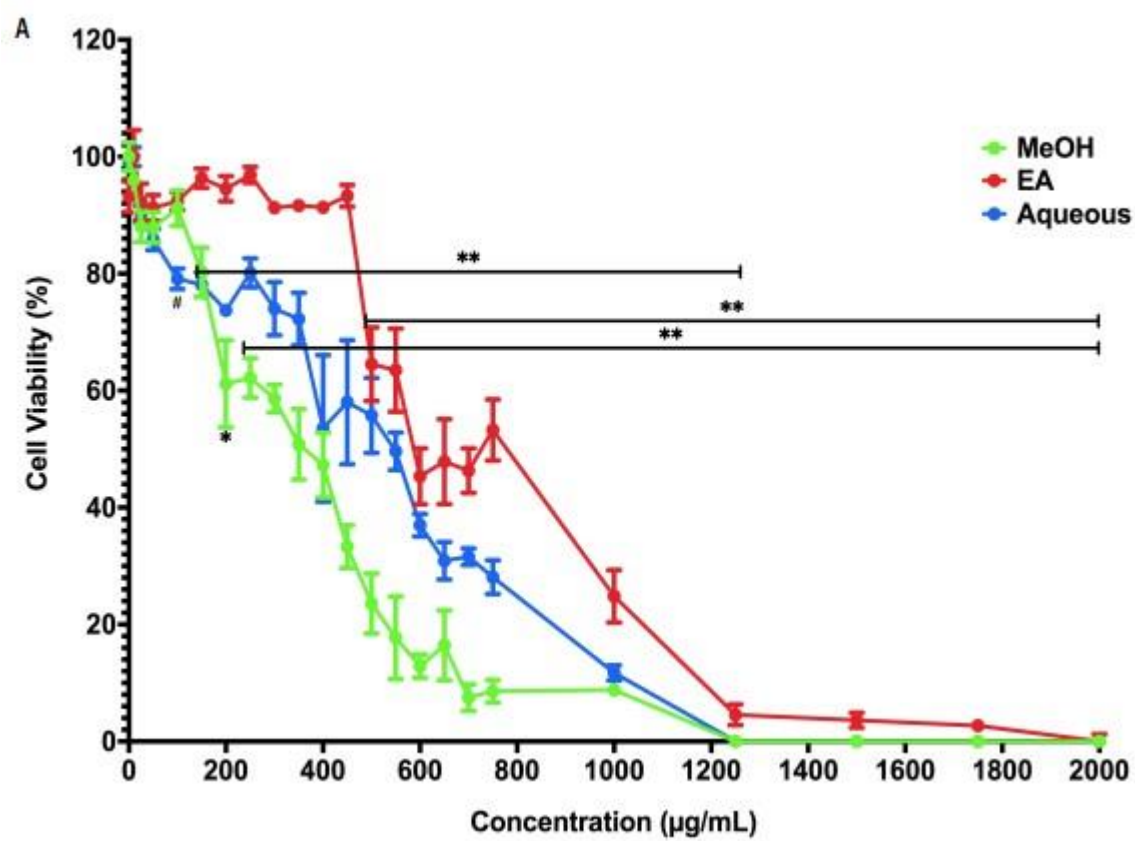


Figure 19. A-B-C) Microscope image of 3T3-L1 adipose cells by MTT stained (10X, 20X and 40X, respectively)



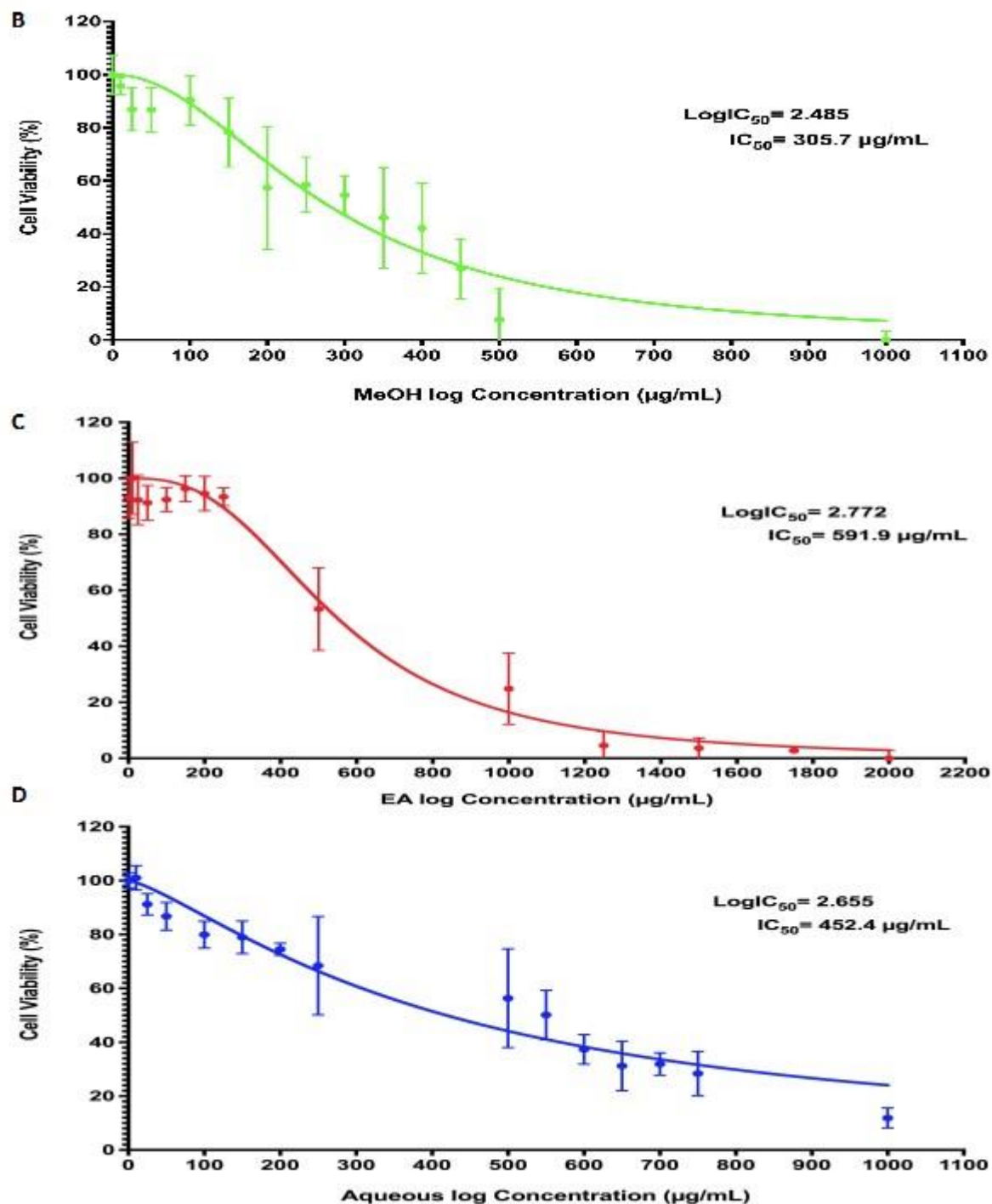


Figure 20. MTT assay showing the effect

B. spargulifolius extracts on 3T3-L1 cell viability and IC_{50} value of the *B. spargulifolius* extracts. **A.** Effect of *B. spargulifolius* extracts on 3T3-L1 cell viability. The mature 3T3-L1 adipocytes were treated for 24 h with increased concentration of Methanol (MeOH), Ethylacetate (EA), and Aqueous extracts of *B. spargulifolius*. Also, cell treated without crude plant extract was taken as control. **B. C. D.** The half-maximal (50%) inhibitory concentration (IC_{50}) value of the *B. spargulifolius* extracts. The IC_{50} is calculated by non-linear regression model. Data expressed as the means $\pm$ SD from three independent experiments. To analyse statistical was used One-way ANOVA test. * $P = 0.033$, # $P = 0.001$ and ** $P < 0.001$, significantly different from control.

4.3. Effect of *B. spergulifolius* extracts on cell viability in 3T3-L1 adipocytes

A live/dead assay was performed to verify the cytotoxic effect of *B. spergulifolius* extracts on the mature 3T3-L1 adipocyte cell line. Cells were exposed to IC₅₀ dosage of MeOH, EA and Aqueous of *B. spergulifolius* extracts for 24 hours. Then, double staining was performed using C-AM and Eth-1 to distinguish between live (green) and dead (red) cells.

Microscopic images of living and dead cells in preadipocytes, adipocytes and mature 3T3-L1 adipocytes treated for 24 hours in staining performed with the live-dead assay were presented in Figure 21A. Under the inverted microscope, intensive green (C-AM) fluorescence was observed in preadipocytes and untreated adipocytes. Red (Eth-1) fluorescence with reduced green (C-AM) fluorescence intensity was observed in mature 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes (Figure 21A).

The percentage of viable cells after 24 hours in preadipocyte and untreated adipocytes was $94.25 \pm 2.98\%$ and $97.5 \pm 1.29\%$ ($P= 0.1836$). However, compared to mature 3T3-L1 adipocytes, after 24 hours treatment of *B. spergulifolius* MeOH, EA and Aqueous extracts, the percentage of viable cells was $69.75 \pm 1.70\%$, $61.75 \pm 1.70\%$ and $70 \pm 4.24\%$ decreased ($P < 0.0001$, $P < 0.0001$ and $P = 0.0014$). Also, compared to Aqueous extract, *B. spergulifolius* MeOH and EA extract treatments resulted in a 0.35% and 11.78% reduction in percentage of viable cells, respectively. (EA-MeOH, EA-Aqueous and MeOH-Aqueous respectively, $P = 0.0122$, $P = 0.0154$ and $P = 0.9157$) (Figure 21B).

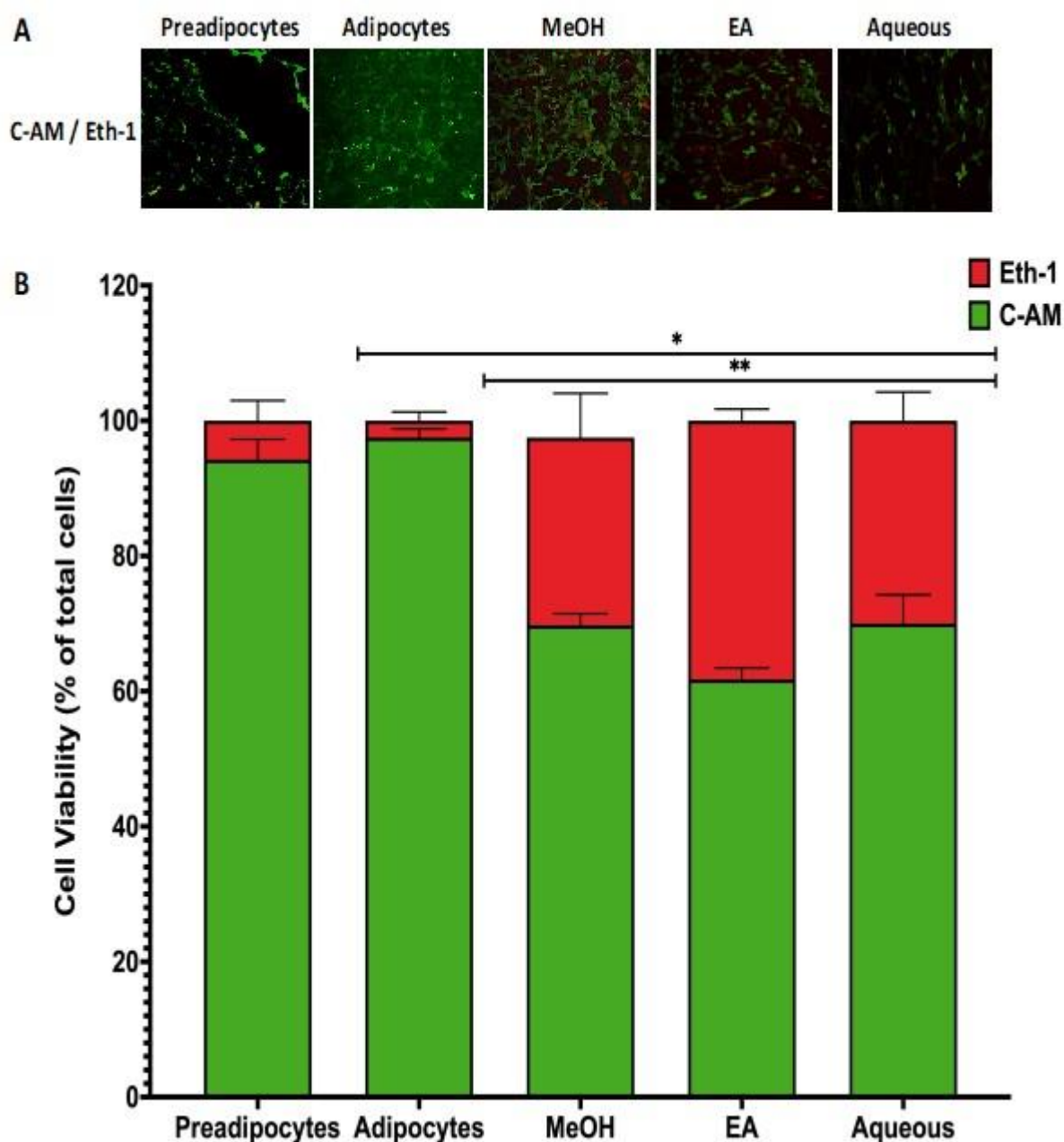


Figure 21. Live-dead staining showing the effect

B. spargulifolius extracts on cell viability on in 3T3-L1 adipocytes. Live-dead staining assay was performed preadipocytes, mature adipocytes without and mature adipocytes with treated with IC₅₀ dose of MeOH, EA and Aqueous of *B. spargulifolius* extracts for 24 h **A**. Representative microscopic images at 10X magnification showed 3T3-L1 cells without and treated with *B. spargulifolius* extracts stained with Calcein-Acetomethoxy (CAM) and Ethidium homodimer-1 (Eth-1). The Eth-1, C-AM (green fluorescence) and Eth-1 (red fluorescence) staining showed respectively live and dead 3T3-L1 cells. **B**. Quantitative data of live-dead staining assay. Three coverslips for each cell group were evaluated. Six distinct-randomly chosen region in each coverslip were visualized and analyzed using microscope and ImageJ software. Data are expressed as a total percentage of viable and dead cells and presented as the means $\pm$ SD. To analyse statistical was used Student's t-test (twotailed). *P < 0.05, significantly different from mature 3T3-L1 adipocytes. **P < 0.05, significantly different from Aqueous.

4.4. 3T3-L1 preadipocyte cells differentiated into mature adipocytes and effect of *B. spergulifolius* extracts on lipid accumulation in 3T3-L1 adipocytes

Oil Red O staining was applied to confirm the adipogenic phenotype in differentiation of preadipocytes into mature 3T3-L1 adipocytes cells and to evaluate the effect of *B. spergulifolius* crude extracts on the lipid accumulation ability of mature 3T3-L1 adipocytes (Akinrinde vd., 2018; Hu vd., 2018).

Staining was performed to distinguish lipid droplets in preadipocytes, adipocytes and adipocytes treated with IC₅₀ dosage of MeOH, EA and Aqueous of *B. spergulifolius* extracts for 24 hours with the Oil Red O kit. Under the inverted microscope, more lipid droplets were observed in untreated adipocytes compared to preadipocytes. Lipid accumulation decreased in mature 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes (Figure 22A).

After Oil Red O staining performing, levels of lipid accumulation were measured using isopropanol. The OD₄₉₀ value increased by $89.23 \pm 6.46\%$ in untreated adipocytes compared to preadipocytes ($P < 0.0001$). As shown in Figure 22B, compared to untreated mature 3T3-L1 adipocytes, 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts decreased in OD₄₉₀ by respectively, $20.2 \pm 2.56\%$, $23.9 \pm 3.16\%$ and $10.3 \pm 2.81\%$ ($P < 0.0001$, $P < 0.0001$ and $P < 0.0016$). In addition, OD₄₉₀ values decreased by 11.09% and 15.20%, respectively, in MeOH and EA extract treatment group adipocytes compared to *B. spergulifolius* Aqueous extract (EA-MeOH, EA-Aqueous and MeOH-Aqueous respectively, $P < 0.0572$, $P < 0.0001$ and $P < 0.0001$) (Figure 22B). Also Oil Red O stained microscope image of oil droplets in mature adipocyte cells is shown in Figure 22.

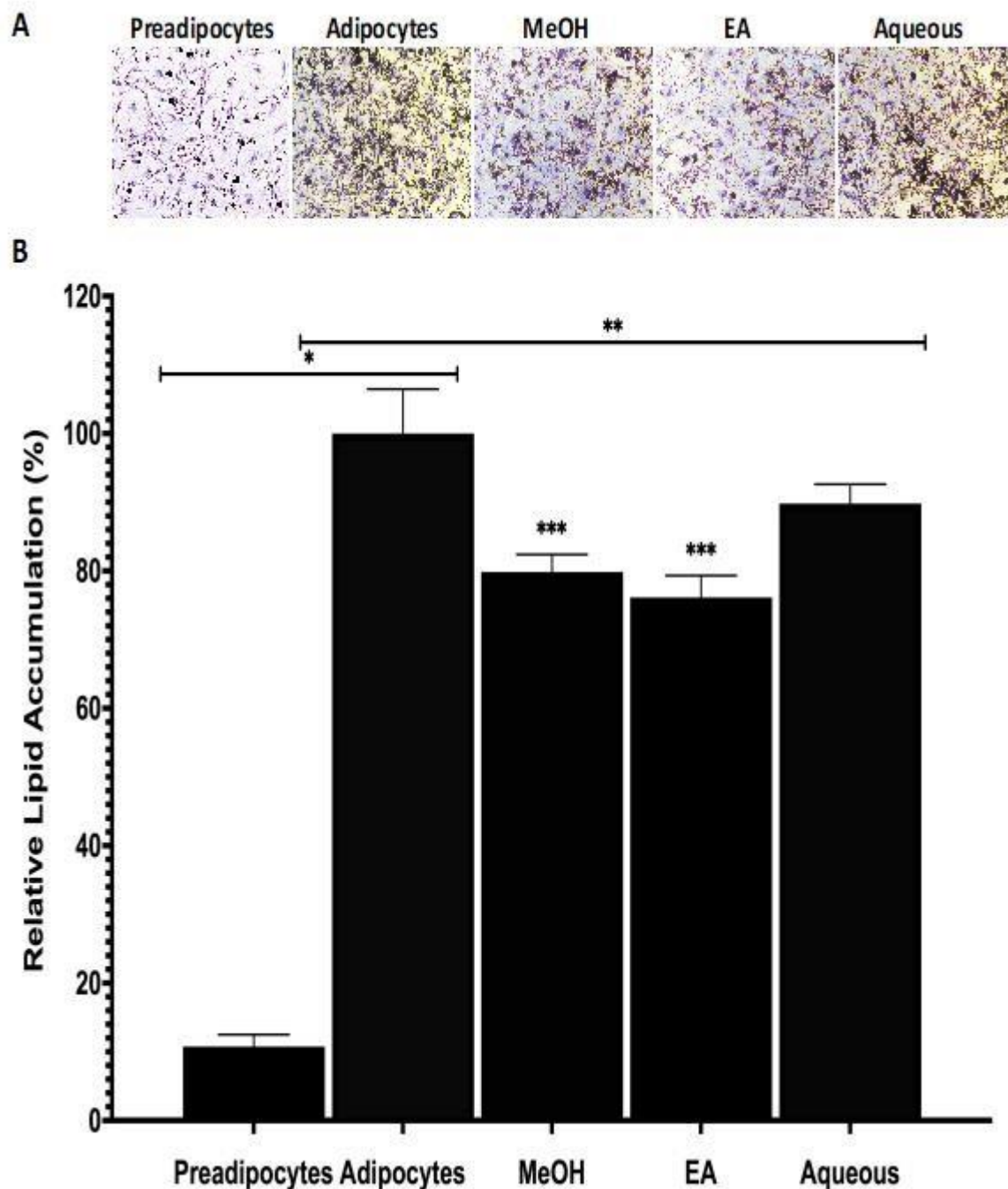


Figure 22. Oil Red O staining showing the 3T3-L1 preadipocyte cells differentiated into mature adipocytes and effects

B. spargulifolius extracts on lipid accumulation in 3T3-L1 cells **A**. The microscopic images at 10X magnification of lipid droplet of preadipocytes, mature adipocytes without *B. spargulifolius* extracts and adipocytes treated with IC₅₀ dose of MeOH, EA and Aqueous of *B. spargulifolius* extracts stained with Oil Red O. **B**. The relative lipid quantization of preadipocytes, mature adipocytes treated without *B. spargulifolius* extracts and with of MeOH, EA, Aqueous of *B. spargulifolius* extracts. OD₄₉₀ of Oil Red O staining from three independent experiments was measured. Data expressed as the relative changing of the OD₄₉₀ and presented as the means $\pm$ SD. To analyse statistical was used One-way ANOVA test. *P < 0.0001 and **P < 0.001, significantly different from mature 3T3-L1 Adipocytes. ***P < 0.001, significantly different from Aqueous.

4.5. Isolated total RNA quantity measurement results

For the synthesis of cDNAs to be used in PCR and quantitative Real time PCR studies, RNA isolation from their cells was first measured in Nanoprop 2000c (Thermo Scientific, USA) device. Results are evaluated according to the nucleic acid concentration (Nucleic Acid. Conc.) and the Abs_{260}/Abs_{280} ratio. *B. Spregulifolius* in the experimental (EA, MeOH, Aqueous) and non-applied control (3T3L1-control) groups, RNA quantity measurements were found at close values between the groups as determined IC_{50} doses of *B. spregulifolius* extracts were applied.

4.6. Measurement of the amount of synthesized cDNA

Spectrophotometer NanoDrop 2000c (Thermo Scientific, ABD) was used for concentration measurements of synthesized cDNAs. During the measurement, cDNAs were kept in ice. For measurements, the water was read as "Blank" in the DNA mode of the device. Abs measurement was made at 260 nm and 280 nm wavelengths using 2 μ L of the dissolved cDNA for cDNA concentration. The cDNAs that were measured were stored at $-80^{\circ}C$ (Nauire, UK).

4.7. Effect of *B. spregulifolius* extracts on apoptosis regulated by the *Bax* and *Bcl-2* in 3T3-L1 adipocytes

qRT-PCR was applied to evaluate the effect of *B. spregulifolius* extracts on *Bax* and *Bcl-2* gene at mRNA levels associated with apoptosis in mature 3T3-L1 adipocytes (Porter and Jänicke, 1999). Cells were treated with IC_{50} dosage of MeOH, EA and Aqueous *B. spregulifolius* extracts for 24 hours. Compared to preadipocytes, *Bax* and *Bcl-2* genes at mRNA level expression was decreased by $23.22 \pm 0.04\%$ and $24.46 \pm 1.70\%$ but *Bax/Bcl-2* ratio increased by $1.654 \pm 0.01\%$, respectively in untreated adipocytes ($P= 0.0015$, $P< 0.0001$ and $P= 0.3451$). Pro-apoptotic *Bax* gene at mRNA level expression increased by respectively, $264.05 \pm 0.9\%$, $347.90 \pm 0.57\%$ and $161.64 \pm 0.30\%$ in mature 3T3-L1 adipocytes treated with *B. spregulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes, while anti-apoptotic *Bcl-2* gene at mRNA level decreased by $15.71 \pm 0.14\%$, $49.01 \pm 0.57\%$ and $18.13 \pm 0.15\%$, respectively (for *Bax*, $P= 0.0020$, $P= 0.0002$, $P= 0.0003$ and for *Bcl-2* $P= 0.0343$, $P< 0.0001$, $P= 0.0423$, respectively). However, compared to *B. spregulifolius* Aqueous extract, an increase in the expression level of the *Bax* gene was 36.14% and 71.18% , respectively, in the MeOH and EA extract treatment groups, while the expression level of the *Bcl-2* gene increased by 2.96% and 37.71% , respectively (MeOH-EA, EA-Aqueous, MeOHAqueous, for *Bax* $P= 0.0919$, $P= 0.0039$, $P= 0.0131$ and for *Bcl-2* $P= 0.0025$, $P= 0.0083$, $P=$

0.6994, respectively) (Figure 23).

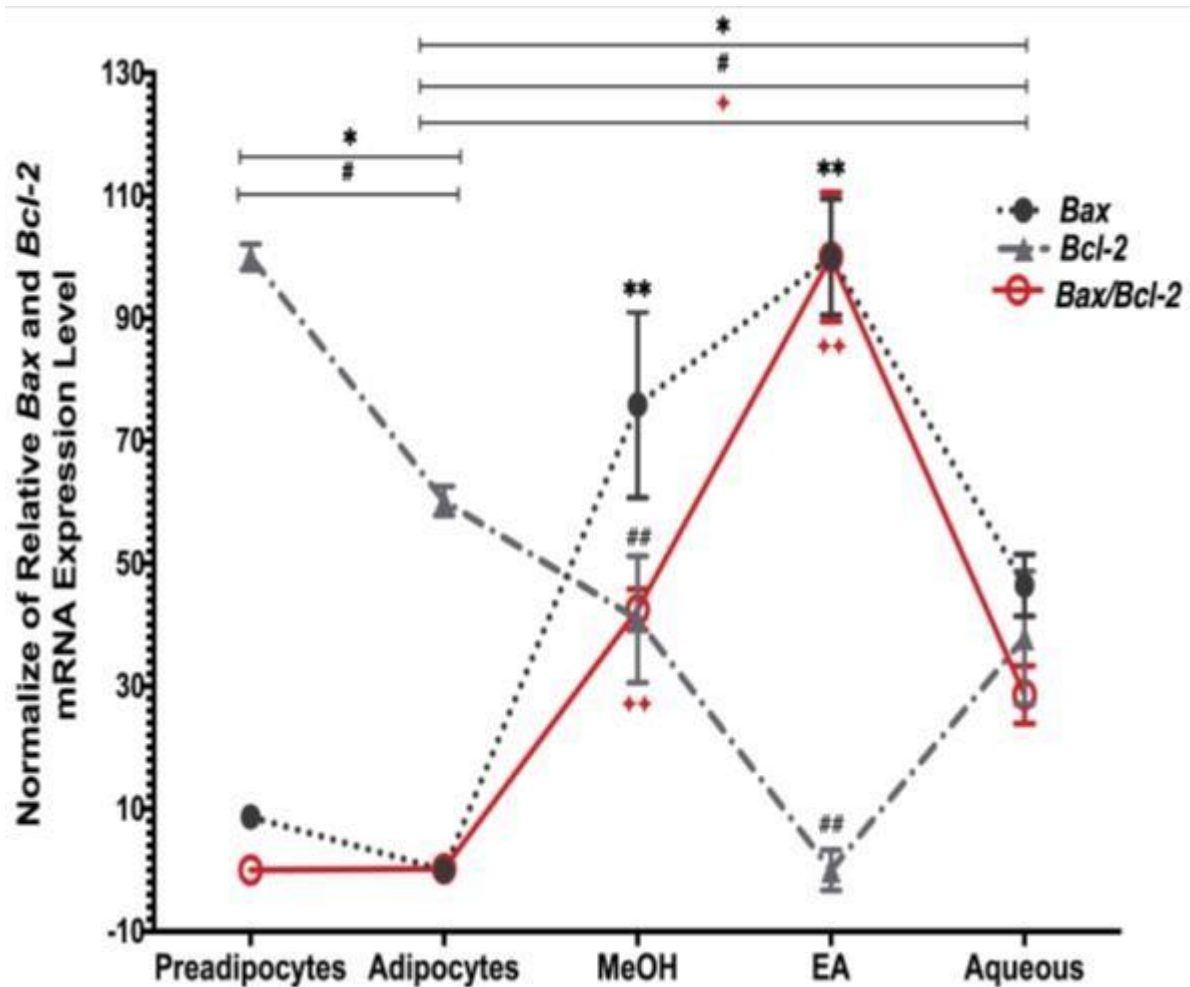


Figure 23. qRT-PCR data showing the effect

B. spargulifolius extracts on apoptosis regulated by the *Bax* and *Bcl-2* in 3T3-L1 adipocytes. The expression at mRNA level of *Bcl-2-associated X* (*Bax*) and *B-cell lymphoma-2* (*Bcl-2*) genes and *Bax/Bcl-2* ratio was detected by qRT-PCR in preadipocytes, mature adipocytes without *B. spargulifolius* extracts and adipocytes treated with IC_{50} dose of MeOH, EA and Aqueous of *B. spargulifolius* extracts for 24 h. The *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) gene were used for normalization. qRT-PCR were analysed using comparative threshold cycle C_T method ($\Delta\Delta C_T$) from three independent experiments. Data expressed as the relative changing of the $\Delta\Delta C_T$ and the means $\pm$ SD. To analyse statistical was used Student's t-test (two-tailed). * $P < 0.05$, # $P < 0.05$ and ** $P < 0.05$, significantly different from mature 3T3-L1 adipocytes. ** $P < 0.05$, ## $P < 0.05$ and *** $P < 0.05$, significantly different from Aqueous.

4.8. Isolated total protein amount measurement results

It was measured with Bradford assay in Nanoprop 2000c (Thermo Scientific, USA) device, primarily by protein isolation from its cells to be used in Western-Blot studies. The measurement results are given in "Figure 46". According to Abs₅₉₅, the protein concentration in each sample was determined as " $\mu\text{g/mL}$ " based on the standard curve was drawn by the

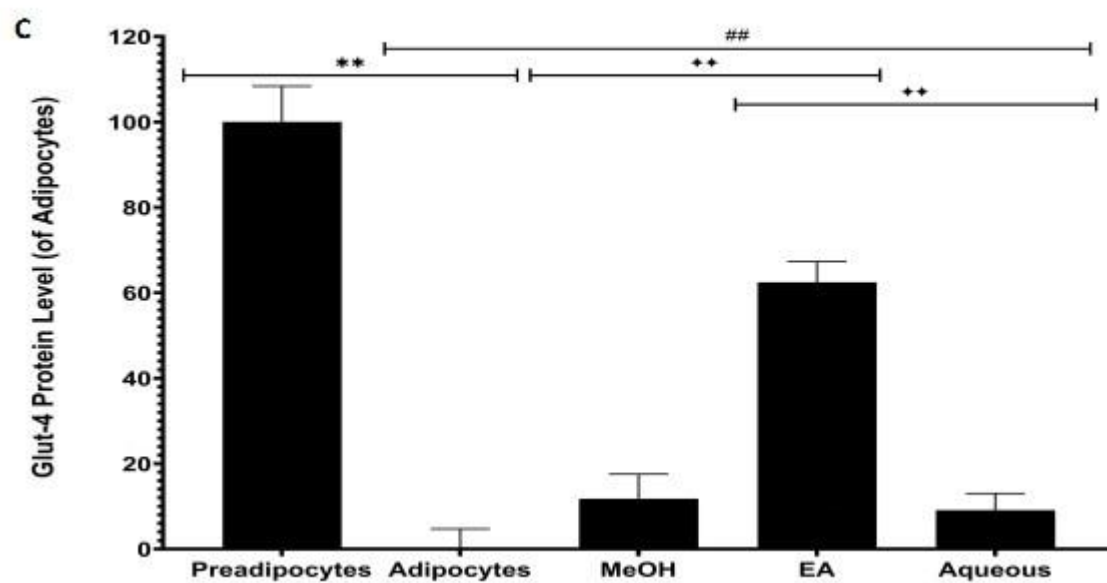
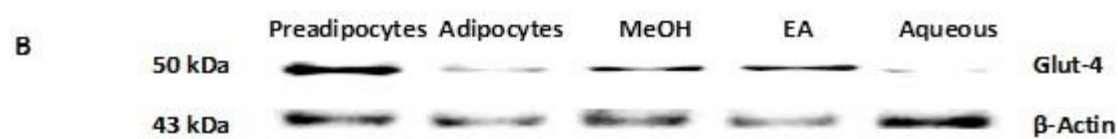
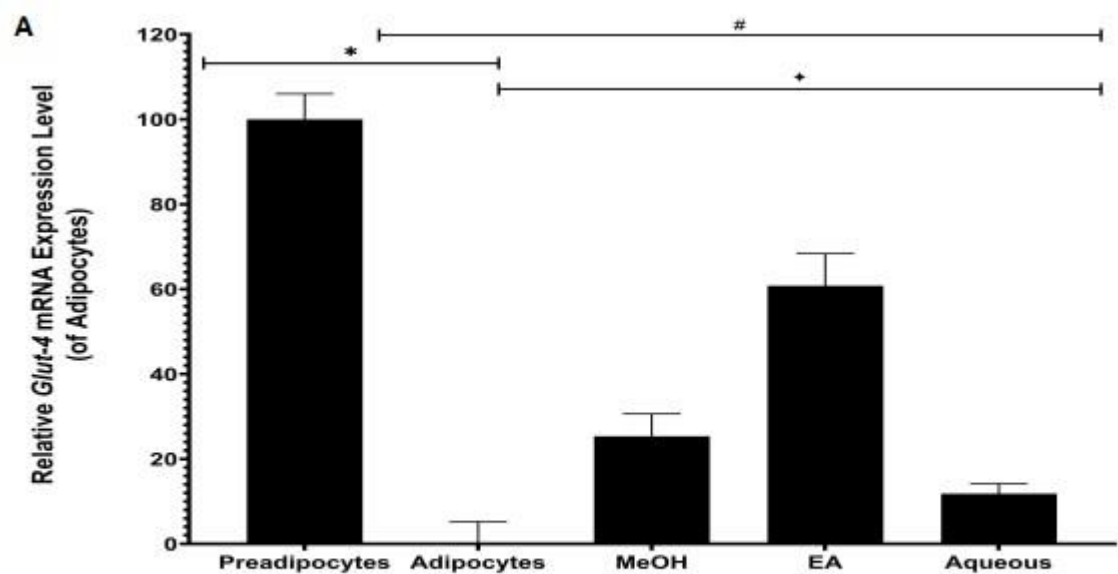
standards ($R^2 = 0.981$). In the experimental and control (groups, protein quantity measurements showed close values between the groups.

4.9. Effect of *B. spergulifolius* extracts on expression of *Glut-4* in 3T3-L1 adipocytes qRT-PCR, Western-blot and Immunofluorescence staining were applied to evaluate the effect of MeOH, EA, Aqueous of *B. spergulifolius* extracts on *Glut-4* expression in mature 3T3-L1 adipocytes associated with insulin resistance and decreased glucose uptake (Epps-Fung et al., 1997). *Glut-4* expression was evaluated in untreated preadipocytes, adipocytes and adipocytes treated for 24 hours with IC_{50} dose of MeOH, EA and Aqueous of *B. spergulifolius* extracts by qRT-PCR, Western-blot and Immunofluorescence staining (Figure 24).

Expression in *Glut-4* mRNA decreased by $389.57 \pm 1.60\%$ in untreated adipocytes compared to preadipocytes ($P = 0.0004$). The mRNA level expression of the *Glut-4* gene increased by $98.84 \pm 1.39\%$, $236.80 \pm 0.53\%$ and $46.18 \pm 0.62\%$, respectively, in mature 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes ($P = 0.0073$, $P = 0.0012$, $P = 0.0458$, respectively). However, among the *B. spergulifolius* extract treatment groups, an increase of 36.02% and 130.40% was observed in the mRNA level expression of the *Glut-4* gene in the MeOH and EA extract treatment groups, respectively, compared to Aqueous extract. (EA-MeOH, EA-Aqueous and MeOHAqueous, for mRNA expression, $P = 0.0021$, $P = 0.0013$, $P = 0.0106$) (Figure 24A).

Western-blot showed a change in the level of *Glut-4* protein expression between preadipocyte, adipocyte and treated adipocyte groups (Figure 24B). The relative intensities of *Glut-4* protein band in membrane were measured as densitometrically. According to densitometric analysis data expression of *Glut-4* protein decreased by $68.93 \pm 9.60\%$ in untreated adipocytes compared to preadipocytes ($P < 0.0001$). Expression of *Glut-4* protein levels increased by $26.11 \pm 11.65\%$, $138.66 \pm 9.80\%$ and $20.17 \pm 7.92\%$, respectively, in mature 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes ($P = 0.0291$, $P < 0.0001$, $P = 0.004$, respectively). However, compared to Aqueous extract, *Glut-4* protein expression levels increased by respectively, 4.93% and 98.59% in the MeOH and EA extract treatment groups (EA-MeOH, EA-Aqueous and MeOHAqueous respectively, $P < 0.0001$, $P < 0.0001$ and $P = 0.3523$) (Figure 24C).

Under the inverted microscope, anti-Glut-4 antibody fluorescence was weak in untreated adipocytes compared to preadipocytes. However, anti-Glut-4 antibody fluorescence density increased in mature 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes (Figure 24D). According to immunofluorescence staining analysis data, anti-Glut-4 antibody fluorescence intensity decreased $59.87 \pm 13.35\%$ in untreated adipocytes compared to preadipocytes ($P < 0.0001$). Compared to untreated adipocytes, anti-Glut-4 antibody fluorescence density increased by $33.28 \pm 8.68\%$, $58.73 \pm 13.46\%$ and $14.09 \pm 6.96\%$, respectively, in mature 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts ($P = 0.0079$, $P = 0.0021$, $P = 0.071$, respectively). However, anti-Glut-4 antibody fluorescence density in adipocytes increased by respectively, 16.813% and 39.12%, in the MeOH and EA extract treatment groups compared to Aqueous extract (EA-MeOH, EA-Aqueous and MeOHAqueous $P = 0.0028$, $P = 0.0007$ and $P = 0.0152$) (Figure 24E).



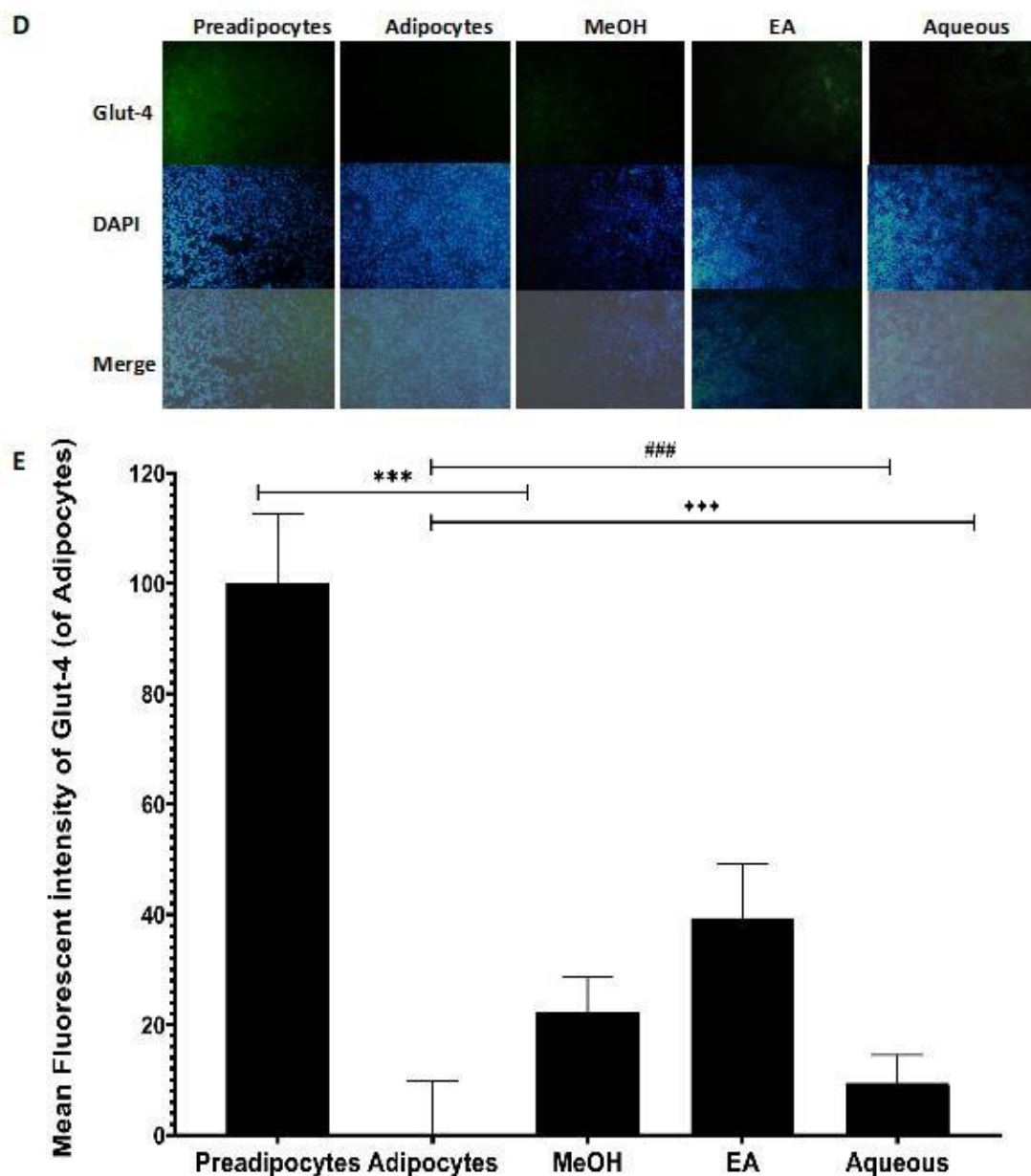


Figure 24. qRT-PCR, Western-blot and immunofluorescence staining showing the effect

B. spargulifolius extracts on translocation and expression of Glut-4 in 3T3-L1 adipocytes. The expression of (Glut-4) gene, protein and intra-cellular were quantified by qRT-PCR, Western-blot and Immunofluorescence staining in preadipocytes, mature adipocytes *without B. spargulifolius* extracts and adipocytes treated with IC₅₀ dosage of MeOH, EA and Aqueous of *B. spargulifolius* extracts for 24 h. **A.** *Glut-4* gene expression was detected using qRT-PCR at the mRNA level. *Gapdh* gene was used for normalization. The relative fold change analysis was conducted by using the $\Delta\Delta CT$ method. qRT-PCR were analysed using $\Delta\Delta CT$ from three independent experiments. Data expressed as the relative changing of the $\Delta\Delta CT$ and presented as the means $\pm$ SD. **B.** Representative image showing Glut-4 protein (55 kDa) levels in 3T3-L1 cells by Western-blot (20 μ g/lane). **C.** The relative intensities of Glut-4 protein band were measured as densitometrically. Beta-Actin (β -Actin) protein was used for normalization. Data was analysed by using the ImageLab software. Densitometric analysis data was presented as the relative changing of the band intensities and the means $\pm$ SD from three independent experiments. **D.** The microscopic image at 10X magnification showing Glut-4 expression levels and nuclear staining in 3T3-L1 cells by Immunofluorescence staining. Merged images obtained with anti-Glut-4 antibody and diamido-2-phenylindole dihydrochloride (DAPI). (Scale bar: 100 μ m **E.** Three coverslips for each cell group were evaluated. Six distinct-randomly chosen region in each coverslip were photographed using microscope and relative intensities of immunofluorescence staining with anti-Glut-4 antibody were quantified using ImageJ software. Immunofluorescence staining analysis data presented as the relative changing of the fluorescein signals intensities and the means $\pm$ SD. To analyse statistical was used Student's t-test (two-tailed). *P < 0.05, **P < 0.0001 and ***P < 0.0001, significantly different from preadipocytes. #P < 0.05, ##P < 0.01 and ###P < 0.001, significantly different from mature 3T3-L1 adipocytes. *P < 0.05, **P < 0.01 and ***P < 0.001, significantly different from among treatment groups.

4.10. The effect of *B. spergulifolius* extracts on the cell migration ability of 3T3-L1 adipocytes

In-vitro scratch assay was applied to evaluate the effect of MeOH, EA, Aqueous of *B. spergulifolius* extracts on the migration of mature 3T3-L1 adipocytes. (Rorth, 2012; Valid, et al 2020). Cell migration and wound closure in untreated preadipocytes, adipocytes and adipocytes treated with IC₅₀ dose of MeOH, EA and Aqueous of *B. spergulifolius* extracts for 24 hours were evaluated with the Scratch assay. The scratch wound was viewed under the microscope at the 0th, 6th, 24th, 30th and 48th hour (Figure 25).

Under the inverted microscope, a large wound area was observed in untreated adipocytes compared to preadipocytes. Compared to untreated adipocytes, treatment group mature 3T3L1 adipocytes treated with the IC₅₀ of *B. spergulifolius* MeOH, EA and Aqueous extracts significantly inhibited wound healing (Figure 25A).

Migration distance to preadipocytes at 0th, 6th, 24th, 30th and 48th hour decreased by $77.52 \pm 2.40\%$, $100 \pm 4.58\%$, $100 \pm 2.00\%$ and $100 \pm 1\%$, respectively (Comparing to previous point of time $P = 0.0003$, $P = 0.0234$, $P = 0.4380$, $P = 0.6220$). Migration distance of adipocytes decreased at 0th, 6th, 24th, 30th and 48th hour $38.07 \pm 2.17\%$, $48.11 \pm 2.03\%$, $99 \pm 2.00\%$ and $100 \pm 0.05\%$, respectively (Comparing to previous point of time, $P = 0.0011$, $P = 0.0002$, $P = 0.0002$, $P = 0.1181$). Migration distance of MeOH decreased at 0th, 6th, 24th, 30th and 48th hour $33.76 \pm 1.63\%$, $34.83 \pm 1.69\%$, $40.24 \pm 1.89\%$ and $51.18 \pm 2.65\%$ respectively. (Comparing to previous point of time, $P = 0.0008$, $P = 0.4590$, $P = 0.0448$, $P = 0.0129$). Migration distance of EA decreased at 6th, 24th, 30th and 48th hour $26.27 \pm 1.59\%$, $26.25 \pm 3.55\%$, $9.50 \pm 1.83\%$ and $7.33 \pm 2.63\%$ respectively (Comparing to previous point of time, $P = 0.0012$, $P = 0.9934$, $P = 0.0035$, $P = 0.4523$). Migration distance of Aqueous decreased at 6th, 24th, 30th and 48th hour $33.96 \pm 2.10\%$, $43.29 \pm 2.96\%$, $43.97 \pm 3.28\%$ and $55.95 \pm 2.25\%$, respectively (Comparing to previous point of time, $P = 0.0013$, $P = 0.0029$, $P = 0.3035$, $P = 0.0209$) (Figure 25B).

The percentage of scratch wound closure was calculated using ImageJ software as the ratio between the scratch-wound area at the initial and at each time point of the assay (Valid, et al 2020). According to data analysis of the percentage of scratch wound closure, Migration distance of untreated adipocytes compared to pre-adipocytes decreased at 6th, 24th, 30th and

48th hour $50.88 \pm 2.17\%$, $51.88 \pm 2.03\%$, $2.94 \pm 2.00\%$ and $0.33 \pm 2.65\%$ respectively ($P=0.0015$, $P=0.0053$, $P=0.3235$, $P=0.4226$). Compared to untreated adipocytes, in the treatment group mature 3T3-L1 adipocytes treated with *B. spargulifolius* MeOH, EA and Aqueous extracts, at 6th hour migration distance decreased by $11.33 \pm 1.63\%$, $30.99 \pm 1.59\%$ and $10 \pm 2.10\%$, respectively. ($P=0.0052$, $P=0.0009$, $P=0.1061$, respectively). In the treatment group 24th hour migration distance decreased by $27.60 \pm 1.69\%$, $45.43 \pm 3.55\%$ and $10.01 \pm 2.96\%$ respectively ($P=0.0108$, $P=0.0130$, $P=0.1167$, respectively). 30th hour migration distance decreased by $59.35 \pm 1.89\%$, $90.40 \pm 1.83\%$ and $55.58 \pm 3.28\%$ respectively ($P=0.0002$, $P=0.0002$, $P=0.0025$, respectively). Also, 48th hour migration distance decreased by $49.49 \pm 2.65\%$, $92.75 \pm 2.63\%$ and $44.78 \pm 2.25\%$ respectively ($P=0.0013$, $P=0.0003$, $P=0.0009$, respectively) (Figure 25B).

In addition, migration distance at 6th hour decreased by 0.60% and 22.63% respectively, in the MeOH and EA extract treatment groups compared to Aqueous extract (EA-MeOH, EAAqueous and MeOH-Aqueous respectively, $P=0.0002$, $P=0.0236$ and $P=0.8878$). Migration distance at 24th hour decreased by 19.54% and 39.36% in the MeOH and EA extract treatment groups compared to Aqueous extract, respectively (EA-MeOH, EA-Aqueous and MeOH-Aqueous respectively, $P=0.1055$, $P=0.0454$ and $P=0.0076$). migration distance at 30th hour decreased by 8.48% and 78.39% respectively, in the MeOH and EA extract treatment groups compared to Aqueous extract (EA-MeOH, EA-Aqueous and MeOH-Aqueous respectively, $P=0.0032$, $P=0.0072$ and $P=0.2028$). migration distance at 48th hour decreased by 8.51% and 86.88% respectively, in the MeOH and EA extract treatment groups compared to Aqueous extract (EA-MeOH, EA-Aqueous and MeOH-Aqueous respectively, $P=0.0004$, $P<0.0001$ and $P=0.0384$) (Figure 25B).

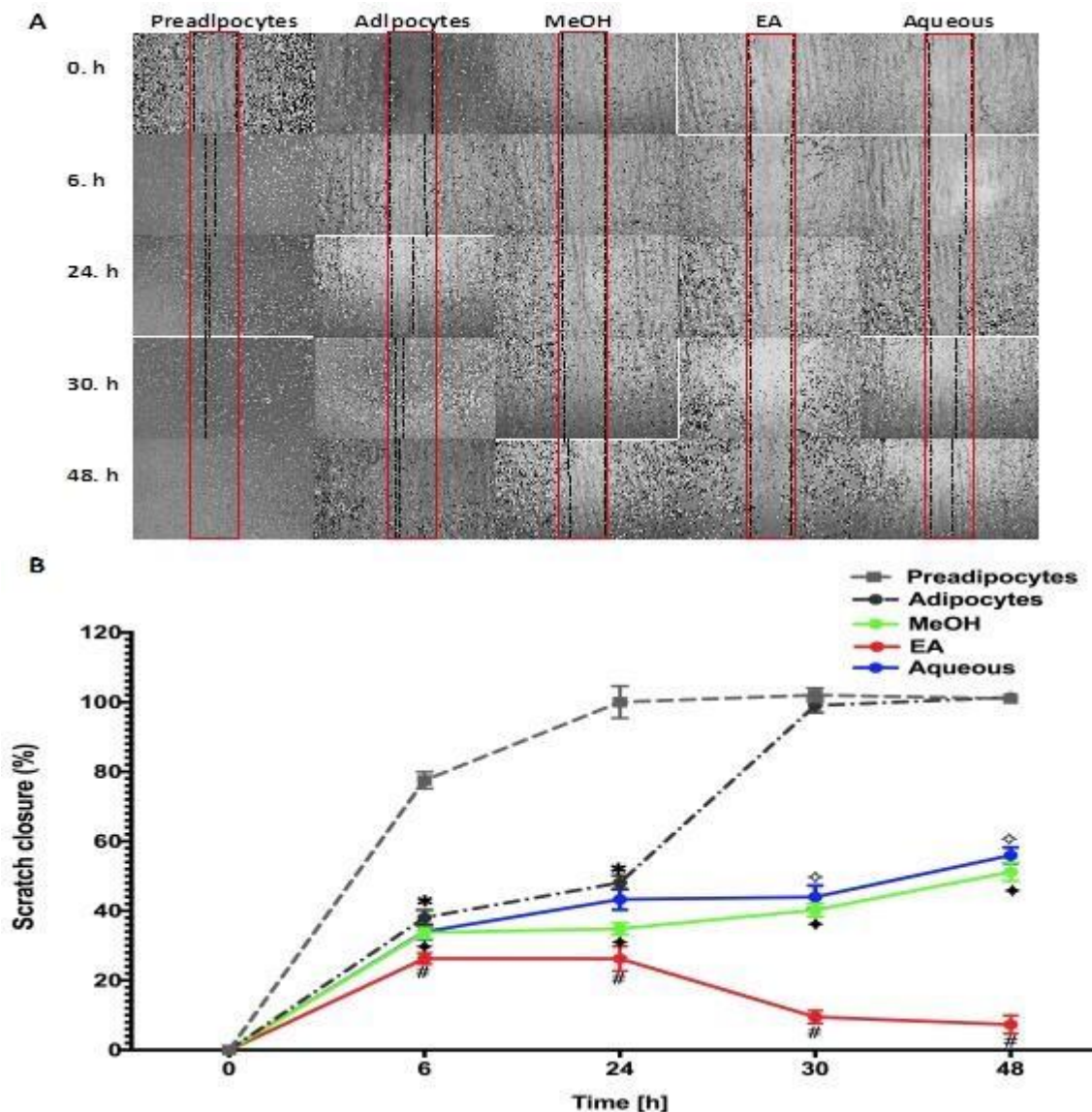


Figure 25. Scratch assay showing the effect

B. spargulifolius extracts on the cell migration ability of 3T3-L1 adipocytes. The cell migration ability was determined by Scratch assay in preadipocytes, mature adipocytes without *B. spargulifolius* extracts and adipocytes treated with IC₅₀ dose of MeOH, EA and Aqueous of *B. spargulifolius* extracts. Cells were cultured to confluent cell monolayers were scratched using the micropipette tip incubated for 0-48 h. **A.** Representative microscopic images (at 10X magnification) captured at 0. h, 6. h, 24. h, 30. h and 48. h showing in-vitro scratch assay wound healing nature of *B. spargulifolius* extracts. The boundaries of the wounds in cells were presented by the red (initial scratch wound) and black (final scratch wound) coloured lines (Scale bar: 100 μ m) **B.** The scratch closure rate was quantified using ImageJ software. Data presented as a percentage of the closure of scratch-wound area at time 0. h and the means $\pm$ SD from three independent experiments. To analyse statistical was used One-ANOVA tests followed by Tukey's test were used. *P < 0.05, significantly different from preadipocytes. $\diamond$ P < 0.05, $\star$ P < 0.05 and $\#$ P < 0.05, significantly different from mature 3T3-L1 adipocytes.

4.11. Effect of *B. spargulifolius* extracts on the cell morphology and diameter of 3T3-

L1 adipocytes

Microscopic examination and SEM, Immunofluorescent and Giemsa staining were applied to evaluate the effect of MeOH, EA, Aqueous of *B. spergulifolius* extracts on morphology in mature 3T3-L1 adipocytes. Cells in untreated adipocytes showed spherical morphology compared to preadipocytes with the phase-contrast microscopy and SEM, Immunofluorescent staining and Giemsa staining. No significant morphologic difference was observed in the treatment group mature 3T3-L1 adipocytes treated with IC₅₀ of *B. spergulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes (Figure 26A). However, cells were observed in less rounded morphology in the *B. spergulifolius* EA extract treatment group compared to the other treatment groups.

The ImageJ software was used for measurement of the average cell diameter in images stained by Giemsa. Cell diameter increased by $139.66 \pm 12.94\%$ in untreated 3T3-L1 adipocytes compared to preadipocytes ($P < 0.0001$). Cell diameter decreased by $41.10 \pm 4.8\%$, $51.74 \pm 6.37\%$ and $30.65 \pm 7.60\%$, respectively in treatment group mature 3T3-L1 adipocytes treated with *B. spergulifolius* MeOH, EA and Aqueous extracts compared to untreated adipocytes ($P < 0.0001$, $P < 0.0001$ and $P < 0.0001$, respectively). In addition, cell diameter decreased 15.06% and 30.40%, respectively in MEOH and EA extracts compared to Aqueous (EA-MeOH, EA-Aqueous and MeOH-Aqueous respectively, $P = 0.0152$, $P < 0.0001$ and $P = 0.0695$) (Figure 26B).

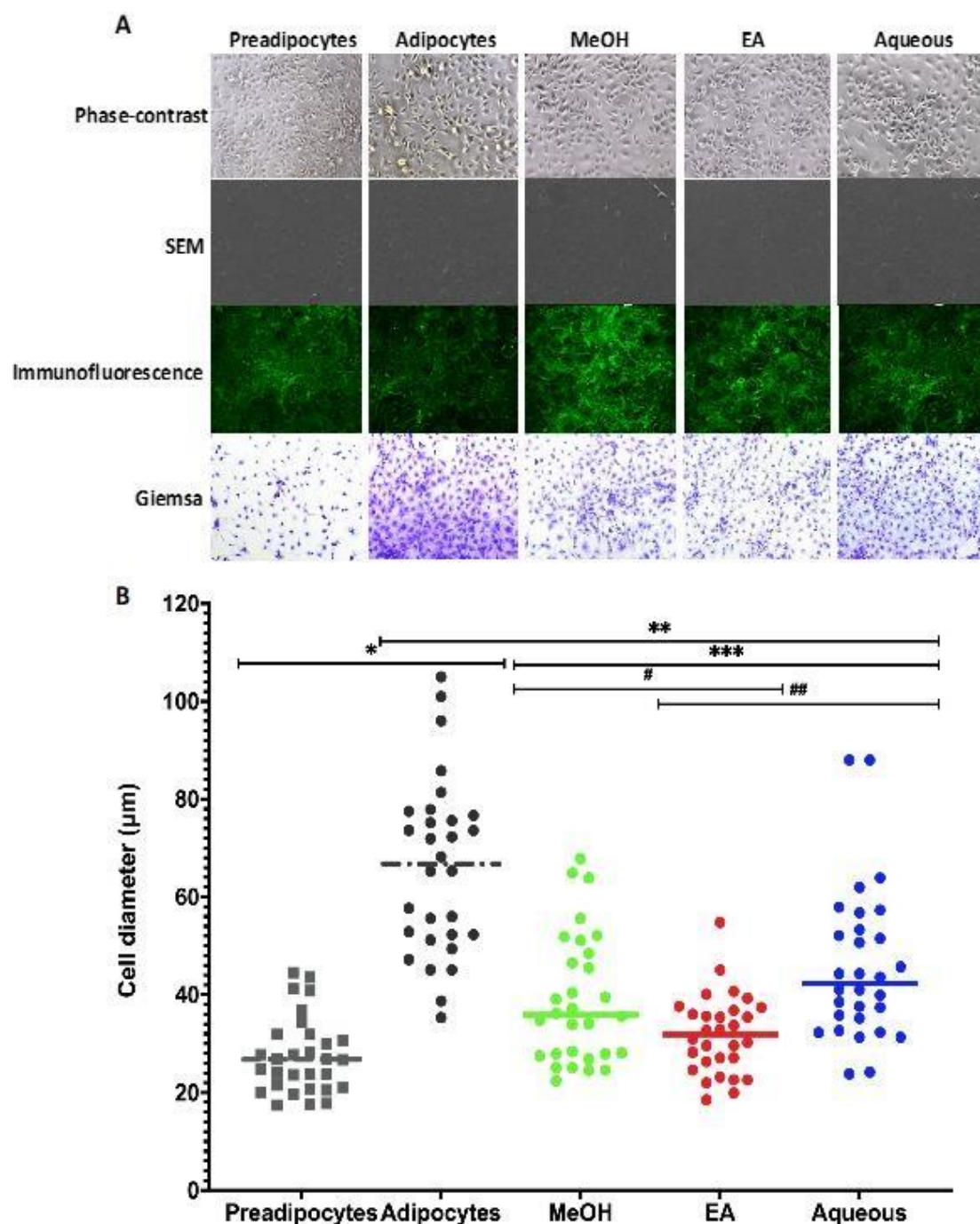


Figure 26. The phase-contrast microscopy, SEM,

Immunofluorescence staining and Giemsa staining showing the effect of *B. spargulifolius* extracts on the cell morphology and diameter of 3T3-L1 adipocytes. **A.** The cell morphology was evaluated by phase-contrast microscopy, Scanning electron microscope (SEM), Immunofluorescence staining and Giemsa staining in preadipocytes, mature adipocytes without *B. spargulifolius* extracts and adipocytes treated with IC₅₀ dose of MeOH, EA and Aqueous of *B. spargulifolius* extracts. Representative images showing changes of cell-morphology after treatment with IC₅₀ dosage of MeOH, EA and Aqueous of *B. spargulifolius* extracts for 24 h. The cells were imaged under the phase-contrast microscope at 10X magnification (Scale bar: 100 µm). The cells were mounted with thin layer of gold and imaged at 500X using Scanning electron microscope (SEM) (Scale bar: 200 µm). By Immunofluorescence with Phalloidin (Alexa Fluor-488), F-Actin was stained and the cells were viewed under confocal microscopy at 5X magnification (Scale bar: 30 µm). By Giemsa, the cells were stained and viewed under phase-contrast microscope at 10X magnification (Scale bar: 100 µm). **B.** Measurement of cell diameter in images stained by Giemsa. Three wells for each cell group were evaluated. Three distinct-randomly chosen region in each well were imaged under microscope and measurement of the average cell-diameter were quantified using the ImageJ software. Data presented as the average changing of the cell diameter and the means $\pm$ SD. To analyse statistical was used One-ANOVA tests followed by Tukey's test were used. *P < 0.0001, significantly different from preadipocytes. **P < 0.0001, significantly different from mature 3T3-L1 adipocytes. #P = 0.0152 and ##P < 0.0001, significantly different from mature

5. DISCUSSION

DM is a global public health problem and one in two people in the world is unaware that they have diabetes (Cho et al., 2018; Saeedi et al., 2019). T2DM is the most common etiological type of DM, and it adversely affects the health services and economy of developed and developing countries (Forouhi and Wareham, 2014). It is a complex, chronic disease involving dysfunctions in carbohydrate, fat, protein metabolism and /or in the endocrine system in target cell-tissues including reduced daily activity, obesity, adipocyte, pancreatic β cells. Hypoglycemia and glycemic fluctuations in T2DM predispose to the development of pathologies including eye, kidney and neural structures, especially with cardiovascular diseases (Cho et al., 2018). In addition, adipocyte hypertrophy is characterized by the change in adiponectin and adipokine synthesis inducing adipogenesis and lipogenesis. Increased energy demand in adipocytes causes fat accumulation, oxygen demand, insufficient number of mitochondria against low-grade local inflammation, and the development of resistance to insulin, leading to the emergence of the T2DM phenotype (Guilherme et al., 2008). Also, current antidiabetics show various side effects such as hypoglycemia, weight gain and gastrointestinal problems (Dey et al., 2002; Raveendran et al., 2018). Therefore, the discovery and production of safe, natural phyto-pharmaceutical and/or nutraceutical compounds that will target the molecular pathogenesis of T2DM represents new strategies that will contribute to the reduction of underlying risk factors, prevention and or treatment of disease.

Caryophyllaceae species are used by various ethnic groups for medical purposes in diseases such as cough, eye and skin problems, dysentery, headache, heart diseases, diabetes, kidney stones, cataracts, epilepsy, asthma, fever, hypertension and menstrual irregularities (Chandra and Rawat, 2015). It has been reported that the Caryophyllaceae family, which includes the *Bolanthus* (Ser.) Reichb. genus, is a natural source of biologically active compounds for phyto-pharmaceuticals (Zengin et al., 2018). Therefore, in our study, potential anti-diabetic effects of crude extracts obtained from *B. spergulifolius* with three different solvents on mature 3T3-L1 adipocytes were investigated.

Phenolic compounds are powerful scavengers of hydroxyl and peroxy radicals due to their (OH) group content, they are metal binders and have the effect of stopping the Fenton and

Haber-Weiss reactions that are the source of reactive oxygen species (ROS) (Bravo 1998). It has been reported that phenolic compounds taken in the diet play a very important role in reducing oxidative stress and protecting against diabetes, cardiovascular diseases and cancer (Hyun et al., 2016). In addition, it has been stated that phenolic compounds contribute to glycemic control and can be used in DM treatment (Hu et al., 2013; Rasouli et al., 2017). For this reason, in our study, firstly, *B. spargulifolius* was extracted with different solvents. Extraction is an important step in obtaining phytochemicals from the plant. The efficiency of the extraction affect from many parameters such as phytochemicals, the method used and the solvent (Stalikas, 2007). In our study, extracts of *B. spargulifolius* were obtained using MeOH, EA and Aqueous, and the extraction efficiency was observed as 46.91%, 23.2% and 49.3%, respectively. The extraction efficiency increased in relation to the polarity of the solvent used in the extraction. This may be due to the higher solubility of biomachromolecules in Aqueous and MeOH than EA (Zielinski and Kozłowska, 2000). Many assays such as TPC, DPPH scavenging assay, ABTS scavenging assay and Ferric reducing antioxidant power (FRAP) are used to investigate the antioxidant capacity of herbal extracts.

In our study, the total phenolic content of *B. spargulifolius* extracts was investigated by FolinCaicalteu to evaluate the extracellular antioxidant activity (Singleton et al., 1999). TPC values for MeOH, EA and Aqueous were observed as 48.22 mg GAE/g, 52.81 mg GAE/g and 30.34 mg GAE/g *B. spargulifolius*, respectively. The low TPC value in Aqueous extract compared to other solvents may be related to the higher non-phenolic compound content. In addition, the high content of soluble phenolic compounds in TPC value of MeOH and EA can be explained by the fact that they form complex, contain phenolic-containing phenolics and / or these compounds have high molecular weights. Although the Caryophyllaceae family is rich in total phenolic content, the literature on the TPC value of *B. spargulifolius* is quite limited (Zheleva-Dimitrova et al., 2018). Our findings showed that *B. spargulifolius* extracts had a significant phenolic content and MeOH and EA were the better extraction solvent for *B. spargulifolius*.

The MTT test based on the mitochondrial metabolic capacity of living cells was used to evaluate the potential cytotoxicity of *B. spargulifolius* extracts on mature 3T3-L1 adipocyte cells and the suitability of the extracts for further investigation (Mosmann, 1983). After 24 hours incubation with *B. spargulifolius* MeOH, EA and Aqueous extracts, cell viability decreased with an

increase in extract concentration >200 µg/mL, 500 µg/mL, >100 µg/mL, respectively. Moreover, cells that exposed to extract concentrations of 10 µg/mL, 450 µg/mL and ≤25 µg/mL, respectively, maintained over 90% cell viability. IC₅₀ values for *B. spergulifolius* MeOH, EA and Aqueous extracts were observed as 305.7 µg /mL, 567.4 µg /mL and 418.3 µg/mL, respectively. These doses were used in further applications to investigate the potential anti-diabetic effects of the extracts on mature 3T3-L1 adipocytes. Our MTT data showed that *B. spergulifolius* MEOH extract has higher cytotoxic potential than EA and Aqueous extracts. Furthermore, in order to confirm the MTT assay data in our study, the cytotoxic activity of the IC₅₀ dose of the extracts on mature 3T3-L1 adipocytes was evaluated using the live-dead assay (Somaida et al., 2020). Cell viability was observed to be 69.75%, 61.75% and 70% for *B. spergulifolius* MeOH, EA and Aqueous extracts, respectively. These results confirmed that the extracts of *B. spergulifolius* examined were potential cytotoxic agents for the cell line studied.

The 3T3-L1 preadipocyte cell line has the ability to differentiate into the adipocyte phenotype and is a useful in-vitro model that is widely used to study adipogenesis in-vitro (Akinrinde et al., 2018; Hu et al., 2018). When 3T3-L1 preadipocytes in the post-confluent G₀ phase are continuously exposed to an adipogenic factor regimen with various agents, including insulin, they undergo a period of mitotic clonal expansion and then undergo differentiation and growth ceases. The ability of this cell line to differentiate into mature adipocytes is well characterized. The process of adipogenesis includes alteration of cell morphology, growth arrest and clonal expansion, and lipid storage (Green and Kehinde, 1975). With Oil Red O staining, lipid accumulation in the cytoplasm of mature 3T3-L1 adipocytes can be easily observed. (De Melo N, et al. 2019). In our study, it was shown that pre-adipocytes successfully differentiated into 3T3-L1 mature adipocytes after 14 days. In addition, in our study, the effect of *B. spergulifolius* extracts on lipid accumulation in mature 3T3-L1 adipocytes was evaluated with Oil Red O staining. *B. spergulifolius* MeOH reduced the lipid accumulation of EA and Aqueous extracts by 20.2%, 23.9% and 10.3%, respectively. Our data show that *B. spergulifolius* extracts can be used to inhibit lipid accumulation in mature 3T3-L1 adipocytes. Transcriptional activity of some major genes in the insulin signaling pathway such as *Peroxisome proliferator-activated receptor gamma (Pparγ)* and *Glucose transporter-4 (Glut-4)* changes during the maturation of adipocytes to promote the uptake of glucose and fatty acids in adipogenesis. (Hauner et al., 1998; Kim et al., 2012). Glut-4 is responsible for glucose uptake in muscle and adipose tissue

by an insulin-regulated mechanism. (Berenguer et al., 2011). Glut-4 expression increases in response to insulin and the translocation of Glut-4 containing vesicles to the plasma membrane induces glucose uptake into the cell (Reaven, 1988). In T2DM, the expression of Glut-4, which is not stimulated sufficiently due to insulin resistance, decreases, its localization is impaired and glucose transport is prevented in target tissues. (Hu et al., 2013; Xu et al., 2017). Since insulin resistance and decreased Glut-4 expression in adipocyte tissue are characteristic, development of insulin sensitizing agents against T2DM is an important research topic. (Shanik et al., 2008; Epps-Fung vd., 1997). Studies have reported that the expression and translocation of the Glut-4 gene at the mRNA and protein level are reduced in differentiated human adipocyte and in vitro mature 3T3-L1 cells. (Hauner et al., 1998; Tordjman, 1990).

In our study, qRT-PCR, western-blot, and immunofluorescence staining data were consistent, showing that treatment of mature 3T3-L1 adipocytes with *B. spergulifolius* crude extracts caused a significant increase in expression of the *Glut-4 gene* at the mRNA, protein and intracellular level. Considering the critical importance of Glut-4's activity in adipose tissue and its positive correlation with insulin sensitivity, our data shows that *B. spergulifolius* can be used to reduce insulin-resistance in mature adipocytes.

Although apoptosis in adipocytes is not fully understood, it is known that adipocytes are highly resistant to apoptosis. Studies have reported that adipogenesis in 3T3-L1 adipocytes can be inhibited by apoptosis induction. In this way, it has been demonstrated that adipose tissue mass can be reduced by induction of apoptosis (Prins et al., 1994; Chaiittianan et al., 2017).

Induction of apoptosis in mature adipocytes appears to be a critical strategy in the treatment of many metabolic diseases such as diabetes and obesity. Therefore, we investigated whether *B. spergulifolius* extracts have apoptotic activity in mature adipocytes. Bax/Bcl-2 balance and Caspase-3 activation are known to play a key role in apoptosis. (Porter and Jänicke, 1999). In addition, several natural compounds such as capsaicin, xanthohumol and isoxanthohumol resveratrol and quercetin have been shown to induce apoptosis in 3T3-L1 adipocytes through up-regulation of *Bax* gene and down-regulation of *Bcl-2* gene in mature adipocytes (Yang et al., 2007; Jeong-Yeh et al., 2008; Hsu and Yen 2007).

In our study, the pro-apoptotic *Bax* gene of *B. spergulifolius* MeOH, EA and Aqueous extracts in mature 3T3-L1 adipocytes was 264.05%, 347.90% and 161.64%, respectively upregulated, and the anti-apoptotic *Bcl-2* gene was 15.71%, 49.01% and 18.13%, respectively down-regulated. Based on the relationship between the change in *Bax/Bcl-2* ratio and apoptosis, our result shows that *B. spergulifolius* induces apoptosis in mature adipocytes. In vitro and in vivo models are available to evaluate the wound healing nature of new therapeutic agents. Of these, the scratch assay is a practical, inexpensive and well-established in-vitro model that provides the first evidence of cell migration and wound healing efficacy of potential therapeutic agents. Cell proliferation and migration are essential components of the wound healing process. In the Scratch assay, scratches are created in-vitro, causing the degradation of the monolayer cell layer and loss of cell-cell contact. Cell migration and proliferation is enhanced by the induction of various factors in the wound area over time. (Hulkower and Herber, 2011; Valid, et al 2020). Therefore, in our study, the scratch assay was applied to evaluate the effect of *B. spergulifolius* extracts on cell migration and wound healing capacity in mature adipocytes. Migration and wound healing in the cells were observed for up to 48 hours. At 30th hour, complete wound healing was observed in the wells containing untreated preadipocytes and adipocytes. However, when *B. spergulifolius* extracts were treated with IC₅₀ value, cell migration was slowed in adipocytes and wound healing was generally delayed. *B. spergulifolius* MeOH, EA and Aqueous extracts inhibited migration and healing by 59.35%, 90.40% and 55.58%, respectively, in the wound area, especially after 30 hours. This data shows that *B. spergulifolius* delayed wound healing in mature 3T3-L1 adipocytes. This may be related with the induction of adipogenesis, suppression of proliferation and induction of apoptosis in adipocytes. (Hsiao et al, 2012).

3T3-L1 preadipocytes are morphologically similar to fibroblasts. Induction of differentiation is directly related to increased globular cell morphology, glucose-uptake and lipid accumulation. Adipogenesis and lipogenesis contribute to adipocyte hypertrophy by inducing lipid accumulation, reorganizing the cytoskeleton, losing fibroblastic morphology, resulting in a spherical shape, and increasing cell size (Gregoire et al., 1998). Therefore, in our study, we evaluated the effect of *B. spergulifolius* extracts on cell morphology and cell-diameter in mature adipocytes using phase-contrast, SEM, Immunofluorescence and Giemsa staining. Preadipocytes lost their fibroblastic morphology after differentiation from differentiation and

acquired a spheric form, which is the morphological feature of mature adipocytes. However, in mature 3T3-L1 adipocytes, extracts of *B. spargulifolius* MeOH, EA and Aqueous had a negative effect on the diameter of the cells and suppressed 41.10%, 51.74% and 30.65%, respectively. Our data, *B. spargulifolius* may contribute to the reduction of the cell diameter and gaining fibroblastic morphology by reducing lipid accumulation in mature adipocytes.



6. CONCLUSION

In our study, we extracted the dried above ground structures of *B. spergulifolius* with MeOH, EA and Aquaeous solvents: this plant was extracted for the first time. We evaluated the total phenolic content of these extracts with Folin-Caiocalteu and observed that the plant may have a significant antioxidant capacity. For further studies, we differentiated 3T3-L1 preadipocytes into mature adipocytes. We evaluated the cytotoxicity of the extracts in mature adipocytes with the MTT assay and determined the IC₅₀ value of the extracts. Consistent with MTT data, we observed that live / dead assay extracts could be potential cytotoxic agent candidates. The effect of the extracts preventing lipid accumulation in 3T3-L1 adipocytes by applying Oil Red O staining; We evaluated it photographically and quantitatively. The extract treatment inhibited lipid accumulation. We evaluated the Glut-4 expression of the extracts in mature adipocytes photographically and quantitatively by qRT-PCR, western-blot analysis and immunofluorescent staining. Extract treatment increased the expression of the *Glut-4* gene at the mRNA, protein, and intracellular level. In addition, we quantitatively evaluated the expression of *Bax* and *Bcl-2* genes in mature adipocytes at mRNA level by qRT-PCR. *B. spergulifolius* extracts up-regulated the *Bax* gene and down-regulated the *Bcl-2* gene, changing the *Bax/Bcl-2* ratio. We evaluated the wound healing capacity of the extracts in mature adipocytes photographically and quantitatively using the Scratch-assay. *B. spergulifolius* extracts delayed wound healing. We evaluated the effect of extracts on cell morphology and cell-diameter in mature adipocytes photographically and quantitatively using phase-contrast, SEM, Immunofluorescence and Giemsa staining. Extracts suppressed the diameter of mature adipocytes and spherical morphology.

Our study reveals the in vitro bioactive capacity of the plant by showing that *B. spergulifolius* extracts reduce lipid accumulation, delay wound healing, induce apoptosis and increase *Glut4* expression. These preliminary data can also provide a basis for the development of phytopharmaceutical strategies aimed at the discovery of components to be isolated from *B. spergulifolius* and the acquisition of products. All of our results show that *B. spergulifolius* can improve its functional capacity in the future and thus play an important role in the prevention and / or treatment of T2DM.

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