http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 25(1): 11-19, 2024
ISSN 2528-9691
DOI: 10.23902/trkjnat.1378819

Research Article

Quantitative HPLC analysis and isolation of tiliroside from *Alcea biennis* Winterl and determination of total flavonoid content and biological activities

Sıla Özlem Şener[1], Tuğba Subaş[2*], Şeyda Kanbolat[3,4], Merve Badem[3], Ufuk Özgen[2], Merve Tamer[2], Mahi Nur Kaplan[2], Esen Sezen Karaoğlan[5]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Health Sciences, Ankara, TÜRKİYE
[2]Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, TÜRKİYE
[3]Department of Biochemistry, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, TÜRKİYE
[4]Health Sciences Institute, Karadeniz Technical University, 61080, Trabzon, TÜRKİYE
[5]Department of Pharmaceutical Botany, Faculty of Pharmacy, Atatürk University, Erzurum TÜRKİYE

Cite this article as:

Şener S.Ö., Subaş T., Kanbolat Ş., Badem M., Özgen U., Tamer M., Kaplan M.N. & Sezen Karaoğlan E. 2024. Quantitative HPLC analysis and isolation of tiliroside from *Alcea biennis* Winterl and determination of total flavonoid content and biological activities. *Trakya Univ J Nat Sci*, 25(1): 11-19, DOI: 10.23902/trkjnat.1378819

Received: 21 October 2023, Accepted: 28 December 2023, Online First: 02 February 2024, Published: 15 April 2024

Edited by:
İpek Süntar

Corresponding Author:
Tuğba Subaş
tugbasubas@ktu.edu.tr

ORCID iDs of the authors:
SÖŞ. orcid.org/0000-0001-7679-7165
TS. orcid.org/0000-0002-0956-6567
ŞK. orcid.org/0000-0001-7261-7067
MB. orcid.org/0000-0002-1265-5616
UÖ. orcid.org/0000-0001-9839-6717
MT. orcid.org/0009-0006-7237-4804
MNK. orcid.org/0009-0000-1391-1530
ESK. orcid.org/0000-0002-9098-9021

Key words:
Alcea
Chromatography
Obesity
Pancreatic lipase
Tiliroside

Abstract: Natural products are being investigated to obtain potential agents that could be used to prevent and ameliorate obesity and oxidative stress, which are two important factors that cause many metabolic disorders in the body. This study aimed to isolate and quantify the major compounds, to determine total flavonoid content, and to evaluate biological activities (antioxidant and pancreatic lipase (PL) inhibitory activity) of the methanol extract and the chloroform, ethyl acetate and the remaining aqueous subextracts of *Alcea biennis* Winterl. The major compound isolated from the ethyl acetate subextract was determined as tiliroside (kaempferol 3-*O*-*β*-(6″-*O*-*trans*-*p*-coumaroyl-glucopyranoside) using 1D-NMR and 2D-NMR spectral methods. The quantification of tiliroside in the extracts was specified by the newly validated high-performance liquid chromatography (HPLC) method. Ferric-reducing antioxidant power (FRAP) assay was performed to determine the antioxidant activity and the total flavonoid content was determined. The ethyl acetate subextract was found to have the highest tiliroside (75.4263 mg/g) and total flavonoid content (189 mg QE/g) and showed the highest FRAP value (259 ± 5.1316 µM TE/g). The methanol extract had a higher PL inhibitory activity (IC_{50} = 100.891 ± 5.160 µg/mL) compared to the other extracts. Tiliroside exhibited higher activity (IC_{50} = 86.203 ± 6.323 µg/mL) than the extracts. It has been demonstrated that *A. biennis* may be a promising plant for treatment of obesity, and further studies should be conducted to evaluate it as a therapeutic agent.

Özet: Vücutta birçok metabolik bozukluğa neden olan iki önemli faktör olan obezite ve oksidatif stresin önlenmesi ve iyileştirilmesi için doğal ürünler araştırılmaktadır. Bu çalışmada, *Alcea biennis* Winterl metanol ekstresi ile kloroform, etil asetat ve arta kalan sulu alt ekstrelerinin ana bileşiklerinin izole edilmesi ve miktarının belirlenmesi, toplam flavonoit içeriğinin tayini ve biyolojik aktivitelerinin (antioksidan ve pankreatik lipaz (PL) inhibitör aktivite) değerlendirmesi amaçlanmıştır. İzolasyon çalışmaları sonucunda etil asetat alt ekstresinden izole edilen ana bileşiğin 1D-NMR ve 2D-NMR spektral yöntemleri kullanılarak tilirozit (kemferol 3-*O*-*β*-(6″-*O*-*trans*-*p*-kumaroil-glukopiranozit) olduğu saptanmıştır. Ekstrelerdeki tilirozit miktarı yeni geliştirilen yüksek performanslı sıvı kromatografisi (HPLC) yöntemiyle belirlenmiştir. Antioksidan aktiviteyi belirlemek için ferrik-indirgeyici antioksidan güç (FRAP) analizi yapılmış ve ayrıca toplam flavonoit içeriği belirlenmiştir. Etil asetat alt ekstresinin en yüksek tilirozit (75,4263 mg/g) ve toplam flavonoit (189 mg QE/g) içeriğine sahip olduğu ve en yüksek FRAP değerini (259 ± 5,1316 µM TE/g) gösterdiği tespit edilmiştir. Metanol ekstresi diğer ekstrelerle karşılaştırıldığında daha yüksek PL inhibitör aktivitesine (IC_{50} = 100,891 ± 5,160 µg/mL) sahiptir. Tilirozit ise ekstrelerden daha yüksek aktivite (IC_{50} = 86,203 ± 6,323 µg/mL) sergilemiştir. *Alcea biennis*'in obezite tedavisi için umut verici bir bitki olabileceği ortaya konulmuştur ve terapötik ajan olarak değerlendirilmesi için daha ileri çalışmalar yapılmalıdır.

Introduction

Obesity and overweight, which affect all age groups with an increasing prevalence, are defined as excessive and abnormal fat accumulation that harms human health (WHO 2018). Although the number of people affected by obesity and overweight was 2.6 billion in 2020, it is claimed that this number will exceed 4 billion in 2035 (WOA 2023). Since obesity is a serious problem worldwide and the associated diseases increase, therapeutic investigations for the treatment of obesity, which could be consulted in addition to other than lifestyle changes and exercises, still continue. There are different options, such as reducing energy intake, increasing energy expenditure, decreasing fat absorption, and increasing lipolysis, used currently for obesity treatment (Hasim *et al*. 2023). In the case of decreasing fat absoprtion, inhibition of pancreatic lipase (PL) is targeted (Liu *et al.* 2020). Although orlistat, as a PL inhibior, is used in the treatment of obesity, it is difficult to tolerate its side effects, which in turn made natural products an important resource for the discovery of more effective agents with less side effects (Bustanji *et al*. 2011).

The genus *Alcea* L. (Malvaceae) is represented by 70 species in the world and has the highest number of species in Iran, Russia, and Türkiye, respectively. The distribution range of the genus covers Europe (except the north), North of America, Africa, Southern Russia, Anatolia, and Afghanistan (Uzunhisarcikli & Vural 2012). The species of the genus are known as "hatmi", in general, in Türkiye, and *Alcea biennis* Winterl, (syn.: *A. pallida*) in particular, is known with various names such as Gülhatmi, Gülfatma, Fatma çiçeği, Fatmaana otu and sığırkuyruğu (Bulut & Tuzlaci 2013, Sagiroglu *et al*. 2013).

The flowers of *Alcea* species have medicinal uses owing to the large amount of mucilage (Uzunhisarcikli & Vural 2012). For instance, *A. biennis* is used in folk medicine for bronchitis (Polat *et al*. 2013, Tetik *et al*. 2013), asthma (Erarslan *et al*. 2021), conjunctivitis (Tuzlaci & Eryasar-Aymaz 2001), toothache (Sagiroglu *et al*. 2013), wounds and cuts (Polat *et al*. 2013), and as an antitussive (Polat *et al*. 2013, Akyol & Altan 2013, Tetik *et al*. 2013, Erarslan *et al*. 2021, Bagci & Keskin 2022), expectorant (Erarslan *et al*. 2021), ophthalmic (Sagiroglu *et al*. 2013), diuretic (Bulut & Tuzlaci 2013, Erarslan *et al*. 2021), antidiarrheal (Tuzlaci & Erol 1999), and antifungal (Bulut & Tuzlaci 2013).

The study of Ertas *et al.* (2016) showed that *A. biennis* has fatty acids (palmitic acid, linolenic acid), phenolic acids (salicylic acid, ascorbic acid, caffeic acid), and essential oil (arachidic acid, α-selinene, heptacosane, tetratetracontane, hexadecanoic acid) (Ertas *et al*. 2016). It was also reported that the species has antioxidant (Kirca & Arslan 2008, Tusevski *et al*. 2014, Ertas *et al*. 2016), antimicrobial, butyrylcholinesterase inhibitory (Ertas *et al*. 2016), and antistress (Aydin *et al*. 1992) activities.

The total phenolic and flavonoid contents of the species were also evaluated (Tusevski *et al*. 2014, Irtem-Kartal *et al*. 2020), but there has been a few biological activity studies on the species. To our knowledge, there are no isolation studies on major secondary metabolites of *A. biennis*. In the present study, we aimed to isolate the main secondary metabolites from various extracts of the aerial parts of *A. biennis* (ABA) and to quantify them by HPLC. We also determined the total flavonoid contents, antioxidant and PL inhibitory activities of the extracts.

Materials and Methods

Plant Material

Alcea biennis was collected from Hasankale on 10.07.2015, in a locality around the Otlukkapı village of Erzurum province, Türkiye (Location: B8-Latitude/Longitude/Altitude: 39°55.340 N 41°41.704 E, 1683 m). The identification of the plant material was performed by Meryem ŞENGÜL KÖSEOĞLU and by the last author of the study. A voucher specimen of the plant is preserved in Atatürk University, Biodiversity Application & Research Center (AUEF 1164).

Extraction and Isolation

The aerial parts of *A. biennis* (ABA) were used for extractions. Initially, dried and powdered ABA (460 g) were extracted with methanol [(Sigma-Aldrich)-(2 L, three times)]. The combined methanol extracts were filtered and evaporated to dryness. The methanol extract (ABA-M, 49.2 g) was suspended in 300 mL of water:methanol (9:1) mixture. Then the suspension was partitioned with chloroform [(Sigma-Aldrich)-(300 mL, twice)], and the solvent was evaporated using a rotary evaporator to obtain the chloroform subextract (ABA-C, 16.3 g). In the same manner, the water:methanol (9:1) mixture was partitioned with ethyl acetate [(Sigma-Aldrich)-(300 mL, twice)], and after evaporation of the solvent, the ethyl acetate subextract (ABA-E, 1 g) was obtained. The remaining aqueous subextract (ABA-A, 30.3 g) was acquired by evaporating the remaining aqueous phase under reduced vacuum at 40°C.

ABA-E was separated by Sephadex LH-20 (Sigma-Aldrich) column chromatography. Elution was performed with 100% methanol. Fifteen fractions (10 mL each) were collected and fraction 11 gave ABA-E1 (tiliroside, 15 mg). Nuclear Magnetic Resonance (NMR) spectra of APA-E1 was obtained by Bruker Ascend^{TM} 400 MHz/54 mm ULH in CD_3OD.

Thin layer chromatography (TLC, aluminum sheets 20×20 cm, silica gel 60 F_{254}; Merck, 5554) was used to identify the compounds and check their purity. TLC spots were identified by spraying 1% vanillin/H_2SO_4 (Sigma-Aldrich) and then heating at 110°C for 1-2 min. In the studies on ABA-E, $CHCl_3$:MeOH:H_2O (80:20:2, 70:30:3) solvent systems were used for TLC.

Quantitative analysis of ABA-E1 (Tiliroside) by HPLC Method

Preparation of ABA-E1 Solutions

A stock solution including ABA-E1 (100 µL) isolated from ABA-E was prepared and dissolved in HPLC-grade methanol. The prepared solution was filtered through a 0.45 µm membrane filter. The stock solutions of ABA-E1 were diluted between 7.5-100 µg/mL and used to create a calibration curve.

Preparation of Sample Solutions

To determine the ABA-E1 content by HPLC (Shimadzu Corporation, LC 20 AT, Kyoto, Japan) analysis, ABA-M, ABA-C, ABA-E, and ABA-A solutions were prepared.

HPLC Conditions

The newly validated HPLC method was applied for analysis. For this purpose, a C18 column (4.6 × 150 mm, 5 µm) and a two-solvent gradient system (100% acetonitrile B: ~ 2.5% (v/v, adjust to pH 2.75) acetic acid in deionized water, constant solvent flow rate 1.2 mL/min) with a diode array detector between wavelengths 240 and 360 nm was used. The gradient program was as follows: 0.01 min 45% A, 55% B; 1 min 45% A, 55% B; 2 min 50% A, 50% B; 4 min 50% A, 50% B; 5 min 60% A, 40% B; 7 min 60% A, 40% B; 8 min 80% A, 20% B; 9 min 80% A, 20% B; 10 min 90% A, 10% B; 20 min 90% A, 10% B.

Method validation

Validation of the method was appraised for linearity, recovery, precision, and selectivity according to ICH guidelines (EMA 2022). Linearity was evaluated with calibration curves containing five different ABA-E1 concentrations ranging from 7.5-100 µg/mL and five replicate data points. To obtain a linear regression equation and find the correlation coefficient, peak areas were plotted over different ABA-E1 concentration ranges (Table 1). Recovery, percent concentration of ABA-E1 in triplicate was investigated for three different concentrations. Recovery was expressed as the mean and standard deviation of the known amount in percent. Three different concentrations of ABA-E1 were measured intra-day and inter-day to assess precision. The relative standard deviation (%RSD) of the retention times for two separate days and the percentage of peak areas were determined. The selectivity of the method was evaluated by comparing the chromatograms.

Quantitative analysis

ABA-E1 content in the extract and subextracts was found on the obtained calibration curve. Each sample was run in triplicate by the new HPLC method according to the quantitative analysis specification.

Determination of Total Flavonoid Content

The total flavonoid content (TFC) of the extracts was determined by the aluminum trichloride method. 1 mL of extract (2 mg/mL) and 1 mL of 2% $AlCl_3$ were mixed, and for all samples, a mixture of 1 mL of extract and 1 mL of methanol was used as blind. After the samples were kept at room temperature for 10 minutes, absorbance values were determined at 415 nm against the blank using a spectrophotometer (BMG Labtech Spectrostar Nano). The flavonoid content of the samples was found as the quercetin equivalent (expressed as mg QE/g) by the equation of the curve obtained from the absorbance concentration graph of the quercetin (standard) (Arvouet-Grand *et al*. 1994).

Determination of Antioxidant Capacity

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay is based on measuring the ferric-reducing capacity of the samples. Extracts were prepared at a concentration of 10 mg/mL. 50 µL of the sample solution and 1.5 mL of freshly prepared FRAP reagent were pipetted onto the standard blank. Samples' own solvents were used as blanks. After vortexing, the tubes were incubated at room temperature for 20 min. Later, absorbance values were read at 595 nm using a spectrophotometer (BMG Labtech Spectrostar Nano). The FRAP test results were found as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich) equivalent with the help of the curve prepared with the absorbance concentration values of Trolox used as a standard. The results were determined as µM Trolox per g sample (TEAC) (Benzie & Strain 1999).

Pancreatic Lipase (PL) Inhibition

PL inhibition was evaluated by the method of Bustanji *et al*. (2011) where *p*-nitrophenyl butyrate (*p*-NPB) (Sigma-Aldrich) was used as the substrate. Prepared extracts and orlistat (positive control) were diluted with buffer solution (0.1 M Tris-HCl buffer, pH = 8.0) at different concentrations (12.5-400 and 6.25-100 µg/mL, respectively). Experimental microplate wells were prepared: A: 90 µL enzyme solution [(PL from porcine, type II (Sigma, EC 3.1.1.3)-(200 units/mL)] 5 µL substrate solution (10 mM), 5 µL buffer solution; B: 90 µL of enzyme solution (200 units/mL), 10 µL of buffer solution; C: 90 µL enzyme solution (200 units/mL), 5 µL sample solution, 5 µL substrate solution (10 mM); D: 90 µL enzyme solution (200 units/mL), 5 µL sample solution, 5 µL buffer solution.

Table 1. Validation parameters of the new HPLC method for ABA-E1 (Tiliroside).

Retention Time (min)		**Regression coefficient (R^2)**	
5.38		0.9997	
Retention Time (%RSD)		**Peak Area (%RSD)**	
Intra-day	**Inter-day**	**Intra-day**	**Inter-day**
0.056	0.059	0.27	0.39
% Recovery (Mean ± SD)			
12.5 µg/mL	**50 µg/mL**	**100 µg/mL**	
98.10 ± 0.8185	100.93 ± 0.7505	100.56 ± 0.5773	

RSD: Relative Standard Deviation

Before adding the substrate, the microplates were incubated at 37°C for 15 minutes, then the substrate solutions were added to the wells, and the microplates were incubated again at 37°C for 15 minutes. The microplates were read with a microplate reader at 405 nm (BMG Labtech Spectrostar Nano) and the absorption values were placed in the equation given below, and the % PL Enzyme Inhibition values were calculated. All samples were run in 3 parallels.

$$\text{Pancreatic Lipase Inhibition (\%)} = \left[\frac{(A-B) - (C-D)}{(A-B)}\right] \times 100$$

The % enzyme inhibition values and the logarithm of the concentration (ordinate and abscissa) it belongs to were placed on the graph and the IC_{50} values of the samples on the PL enzyme were found from the obtained graphic equation.

Results

Isolation of ABA-E1 (Tiliroside)

Tiliroside, a glycosidic flavonoid, was isolated from ABA-E by the isolation studies on ABA (Fig. 1).

***trans*-Tiliroside (Kaempferol 3-*O*-*β*-(6″-*O*-*trans*-*p*-coumaroyl-glucopyranoside):** Yellow amorphous substance; ^{1}H-NMR (400 MHz, CD_3OD): δ 8.0 (*d*, 2H, H-2′ and H-6′, *J* = 8.3), 7.43 (*d*, 1H, H-7‴, *J* = 15.9), 7.32 (*d*, 1H, H-2‴ or H-6‴, *J* = 8.0), 6.81 (*d*, 2H, H-2′ or H-6′), 6.81 (*d*, 2H, H-3‴ or H-5‴), 6.33 (*s*, 1H, H-8), 6.15 (*s*, 1H, H-6), 6.10 (*d*, 1H, H-8‴, *J* = 15.9), 4.31 and 4.21 (2H, H-6″), 3.47-3.50 (*m*, 4H, sugar protons); ^{13}C-NMR (100 MHz, CD_3OD): δ 178.3 (C-4), 167.4 (C-9‴), 164.5 (C-7), 161.6 (C-5), 160.1 (C-4′), 159.8 (C-4‴), 157.9 (C-2), 157.0 (C-9), 145.2 (C-7‴), 133.7 (C-3), 130.8 (C-2′ and C-6′), 129.8 (C-2‴ or C-6‴), 125.6 (C-1‴), 121.3 (C-1′), 114.6 (C-3′ and C-5′), 113.3 (C-3‴ or C-5‴), 113.3 (C-8‴), 104.1 (C-10), 102.5 (C-1″), 98.6 (C-6), 93.4 (C-8), 76.6 (C-3″), 74.4 or 74.3 (C-3″ or C-5″), 70.3 (C-4″), 62.9 (C-6″). ^{1}H-NMR, ^{13}C-NMR, HSQC, and HMBC data (Supplementary Material Figs S1-S4) are similar to data in the literature for tiliroside (Cornelio *et al*. 2017, Devi & Kumar 2020).

Results of HPLC Analysis

The parameters of the new HPLC method for validation are shown in Table 1. The method showed good linearity (at 7.5-100 µg/mL concentration, $R^2 > 0.999$) (Table 1, Fig. 2). Relative standard deviation (RSD) values for intra-day and inter-day precision were determined as 0.27% and 0.39% for peak area and 0.056% and 0.059% for retention time, respectively. The accuracy of the quality controls ranged from 98.10% to 100.93% (RSD < 0.83%) (Table 1).

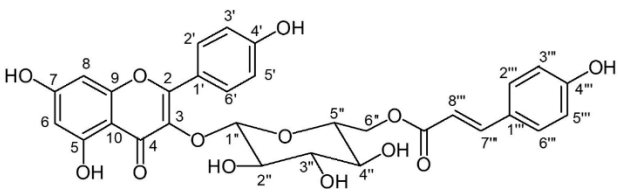

Fig. 1. *trans*-Tiliroside

Table 2. ABA-E1 content detected by HPLC analysis of samples.

Test Extracts	ABA-E1 Content (mg/g crude extract)
ABA-M	40.2508
ABA-C	-
ABA-E	75.4263
ABA-A	16.7311

ABA-M: Methanol extract of *A. biennis* aerial parts (ABA), ABA-C: Chloroform subextract of ABA, ABA-E: Ethyl acetate subextract of ABA, ABA-A: Remaining aqueous subextract of ABA

The quantitative analysis of ABA-E1 was determined according to the peak areas of the samples' chromatograms using the calibration curve (Fig. 2). ABA-E1 contents of all samples were given as mg/g crude extract as a result of HPLC analysis (Table 2, Fig. 2). The highest ABA-E1 content was detected for ABA-E (75.4263 mg/g crude extract), followed by ABA-M (40.2508 mg/g crude extract) and ABA-A (16.7311 mg/g crude extract). It was determined that the HPLC method for the example termed as ABA-C did not contain ABA-E1 in the concentration range.

Total Flavonoid Content

The TFC of the extracts was detected as mg QE/g. As given in Table 3, ABA-E was found to have the highest flavonoid content (mg 189 QE/g).

Antioxidant Activity

The antioxidant activity of the extracts was found by the FRAP assay. FRAP assay results are given as TEAC (µM). ABA-E was found to have the highest FRAP value (TEAC = 259 ± 5.1316 µM) (Table 3).

PL Inhibition

PL inhibitory activities of the extracts and tiliroside are given as IC_{50} in Table 4. Graphs related to enzyme inhibition are presented in Fig. 3. The activities of the extracts and tiliroside are weaker than orlistat. It was observed that ABA-M has more potent activity than other extracts (IC_{50} = 100.891 ± 5.160 µg/mL), and tiliroside showed the highest activity (IC_{50} = 86.203 ± 6.323 µg/mL).

Table 3. TFC and FRAP values of the extracts.

Test Extracts	TFC[a] mg QE/g	FRAP[b] TEAC (µM)
ABA-M	33	22 ± 3.7859
ABA-C	nd[c]	nd
ABA-E	189	259 ± 5.1316
ABA-A	55	125 ± 3.5118

[a]Total flavonoid content expressed in mg of quercetin equivalent (QE) per gram of dry plant weight. [b]FRAP value indicates the ferric-reducing antioxidant power (µM trolox equivalent/gram). [C]: not determined. See Table 2, Fig. 4 for abbreviations.

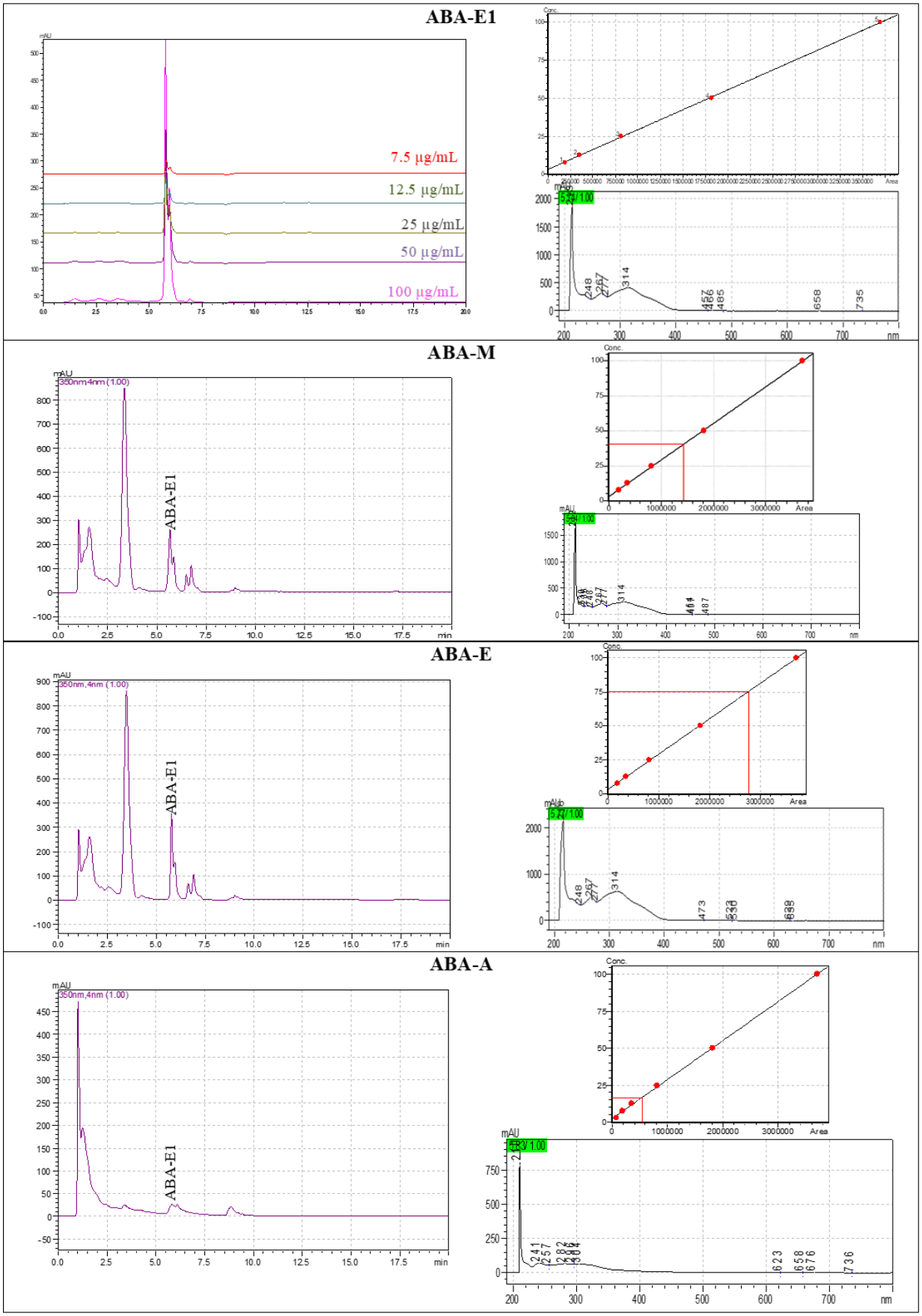

Fig. 2. HPLC chromatograms and calibration curves of the samples.
*ABA-E1: Tiliroside; see Table 2 for abbreviations.

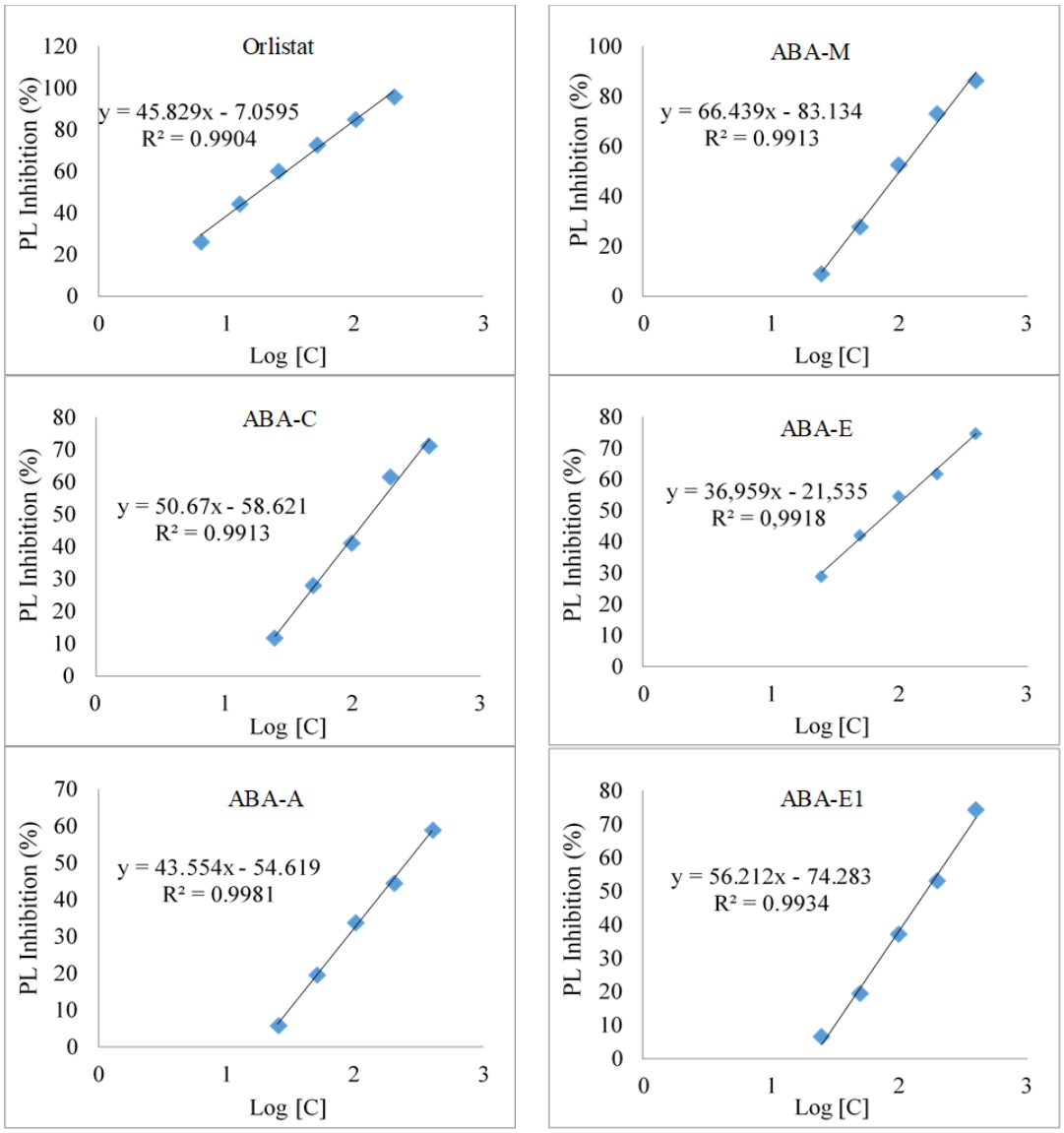

Fig. 3. The graphs of PL inhibition analysis.

Table 4. PL inhibitory activities of the extracts and tiliroside.

Test Compounds	IC_{50} (µg/mL) ± SD
ABA-M	100.891 ± 5.160
ABA-C	139.218 ± 3.309
ABA-E	162.543 ± 4.824
ABA-A	252.379 ± 9.529
Tiliroside	86.203 ± 6.323
Orlistat	17.581 ± 0.714

SD: Standard deviation

Discussion

Obesity (body mass index (BMI) ≥ 30 kg/m^2), which is caused by genetic background of individuals, the environment, irregular diet and inactivity, is a complex condition that causes metabolic disorders such as some types of cancer, type 2 diabetes and cardiac diseases (Bhardwaj *et al*. 2021). As a result of the increasing incidence of obesity worldwide, the investigations for treatment and prevention of obesity still continue. PL inhibition is an important and attractive therapeutic approach for obesity (Buchholz & Melzig 2015). PL is involved in the hydrolysis of triacylglycerols to monoacylglycerols and free fatty acids. After these biomolecules are absorbed in the small intestine, they pass into the blood circulation and reach the adipose tissue and are used in the synthesis of triacylglycerol in adipocyte cells. Since inhibition of PL controls the level of free fatty acid accumulation by delaying/blocking dietary fat absorption, PL inhibitors play an important role in prevention and treatment of obesity (Chia *et al*. 2023). Orlistat, which is currently used clinically for the treatment of obesity and acts as a PL inhibitor, has serious side effects. Natural products, especially plants, are being researched to develop effective and safe new PL inhibitor agents (de la Garza *et al*. 2011). Studies showed so far that

flavonoids, saponins, polyphenols, alkaloids, terpenes and phenolic compounds inhibit PL (Hasim *et al*. 2023).

In the present study, *trans*-tiliroside, which was formerly isolated from *A. rosea* (Abdel-Salam *et al*. 2018) was purified from ABA for the first time. The amount of tiliroside in ABA-M, ABA-E, ABA-C, and ABA-A was determined by the newly developed HPLC method, ABA-E was found to containe the highest amount of *trans*-tiliroside. Tiliroside, consisting of kaempferol, glucose, and *p*-coumaric acid moieties, was determined in fruits, leaves and roots of plants belonging to different families including Malvaceae, Rosaceae and Tiliaceae. Studies demonstrated that it has wide biological activities such as antioxidant, antiinflammatory, neuroprotective, antimicrobial, antiobesity and antiaging (Grochowski *et al*. 2018).

In this study, PL inhibitory activity of tiliroside and total flavonoid content, antioxidant and PL inhibitory activities of the extracts prepared from ABA were evaluated *in vitro*. According to the results of the PL inhibition experiment, ABA-M showed the highest activity compared to other extracts, followed by ABA-C, ABA-E and ABA-A, respectively. It was observed that the PL inhibitory effect of tiliroside was higher than that of the extracts. No study has been performed so far on the PL inhibitory effect of tiliroside, but, there are studies showing that tiliroside is effective for obesity with different mechanisms except for PL inhibition (Ninomiya *et al.* 2007, Goto *et al.* 2012, Nagatomo *et al.* 2013, Nagatomo *et al*. 2015). It was determined that *Rosa canina* L. fruit extract and tiliroside suppress lipid accumulation in adipocytes in part by suppressing peroxisome proliferator-activated receptor-γ (PPAR-γ) expression (Nagatomo *et al*. 2013). In a single-center, double-blind, randomized placebo-controlled study, 32 subjects with a BMI ≥ 25 and <30 were given 100 mg of rosehip extract (*R. canina* extract containing 0.12% tiliroside) or placebo for 12 weeks. Total abdominal fat area, body weight, and BMI were significantly reduced compared to the placebo group. It was concluded that rosehip extract may decrease abdominal visceral fat in preobese individuals and that tiliroside may contribute to this activity (Nagatomo *et al*. 2015). It was determined that *trans*-tiliroside (0.1, 1, and 10 mg/kg) prevented body weight gain in mice, reduced visceral fat, and a single oral administration (10 mg/kg) raised PPAR-α mRNA expression in liver tissue (Ninomiya *et al*. 2007). In an *in vivo* study by Goto *et al*. (2012), it was determined that tiliroside stimulated fatty acid oxidation.

Excessive accumulation of fat in adipose tissue causes the formation of reactive intermediates, decreased cellular defense, and development of oxidative stress, and is therefore thought to be the underlying cause of obesity-related diseases (Manna & Jain 2015). In previous studies, it was determined that total flavonoid content and antioxidant capacity showed a linear relationship (Aryal *et al*. 2019). We showed that ABA-E has the highest flavonoid content and ferric-reducing capacity associated with antioxidant activity. It is known that flavonoids have the potential to show antioxidant activity (Panche *et al.* 2016), and therefore it is an expected result that ABA-E, which has a higher flavonoid content than other extracts, has the highest FRAP value. Ertas *et al*. (2016) determined that the flavonoid content (19.66 ± 1.58 µg QEs/mg) and radical scavenging activity of the acetone extract (at a concentration of 100 µg/mL) was higher than other extracts prepared from the *A. biennis*. The antioxidant activity of tiliroside (the main compound of ABA-E) was investigated by different methods, including FRAP analysis (Ndhlala *et al.* 2013, Li *et al.* 2017, Soltani *et al.* 2023). According to the study of Li *et al*. (2017), the IC_{50} value of tiliroside in the FRAP analysis was found to be 246.8 ± 19.3 µg/mL (550.5 ± 42.9 µM). This value is similar to the IC_{50} value of ABA-E (259 ± 5.1316 µM) in our study. These findings, led us to evaluate tiliroside to contribute to the ferric reducing capacity of the subextract. Using different methods, it has been determined that tiliroside has a radical scavenging effect against reactive oxygen species, and also shows antioxidant activity by inhibiting xanthine oxidase, and increasing superoxide dismutase levels (Grochowski *et al*. 2018). It has been reported that tiliroside (IC_{50} = 1.63 ± 0.86 mg/mL, in TEAC assay) obtained from the ethyl acetate extracts of *Wissedula periplocifolia* (L.) C. Presl and *Sidastrum micranthum* (A.St.-Hil.) Fryxell, both members of Malvaceae, can contribute to the antioxidant activity (Fernandes de Oliveira *et al*. 2012).

In summary, in this study, ABA-E has higher total flavonoid content and higher ferric-reducing capacity than other extracts in relation to its main compound, tiliroside. ABA extracts showed PL inhibitory activity, and tiliroside has the most potent activity. In further studies, the antiobesity effects of ABA can be evaluated in terms of different mechanisms. This study revealed that *A. biennis* is a potential resource that can be evaluated in the preparation of functional products that can be used for prevention and treatment of obesity and its complications.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: The authors confirm that the data supporting the findings of this study are available within the supplementary material of the article.

Author Contributions: Concept: S.Ö.Ş., U.Ö., Design: S.Ö.Ş., T.S., Execution: Ş.K., M.B., M.T., M.N.K., Material supplying: U.Ö., Data acquisition: S.Ö.Ş., T.S., Ş.K., M.B., M.T., M.N.K., Data analysis/interpretation: S.Ö.Ş., T.S., Ş.K., M.B., Writing: T.S., Ş.K., M.B., Critical review: S.Ö.Ş., U.Ö., E.S.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

Funding: The study was supported by the Turkish Scientific and Technical Research Council (TÜBİTAK) with project number 1919B011900478.

References

1. Abdel-Salam, N.A., Ghazy, N.M., Sallam, S.M., Radwan, M.M., Wanas, A.S., ElSohly, M.A., El-Demellawy, M.A., Abdel-Rahman, N.M., Piacente, S. & Shenouda, M.L. 2018. Flavonoids of *Alcea rosea* L. and their immune stimulant, antioxidant and cytotoxic activities on hepatocellular carcinoma HepG-2 cell line. *Natural Product Research*, 32(6): 702-706. https://doi.org/10.1080/14786419.2017.1332602

2. Akyol, Y. & Altan, Y. 2013. Ethnobotanical studies in the Maldan Village (Province Manisa, Turkey). *Marmara Pharmaceutical Journal*, 17: 21-25. https://doi.org/10.12991/201317388

3. Arvouet-Grand, A., Vennat, B., Pourrat, A. & Legret, P. 1994. Standardisation d'un extrait de propolis et identification des principaux constituants [Standardization of propolis extract and identification of principal constituents]. *Journal de Pharmacie de Belgique*, 49(6): 462-468.

4. Aryal, S., Baniya, M.K., Danekhu, K., Kunwar, P., Gurung, R. & Koirala, N. 2019. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants*, 8(4): 96. https://doi.org/10.3390/plants8040096

5. Aydin, S., Ozturk, Y., Baser, K.H.C., Kirimer, N. & Kurtar-Ozturk, N. 1992. Effects of *Alcea pallida* L. (Ae) and *Tilia argentea* Desf. ex DC infusions on swimming performance in mice. *Phytotherapy Research*, 6: 219-220. https://doi.org/10.1002/ptr.2650060411

6. Bagci, Y & Keskin, L. 2022. An ethnobotanical field survey in the Kadınhanı district of Konya in Turkey. *KSU Journal of Agriculture and Nature*, 25(2): 312-336. https://doi.org/10.18016/ksutarimdoga.vi.865880

7. Benzie, I.F. & Strain, J.J. 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, 299: 15-27. https://doi.org/10.1016/s0076-6879(99)99005-5

8. Bhardwaj, M., Yadav, P., Vashishth, D., Sharma, K., Kumar, A., Chahal, J., Dalal, S. & Kataria, S.K. 2021. A review on obesity management through natural compounds and a green nanomedicine-based approach. *Molecules*, 26(11): 3278. https://doi.org/10.3390/molecules26113278

9. Buchholz, T. & Melzig, M.F. 2015. Polyphenolic compounds as pancreatic lipase inhibitors. *Planta Medica*, 81(10): 771-783. https://doi.org/10.1055/s-0035-1546173

10. Bulut, G. & Tuzlaci, E. 2013. An ethnobotanical study of medicinal plants in Turgutlu (Manisa-Turkey). *Journal of Ethnopharmacology*, 149(3): 633-647. https://doi.org/10.1016/j.jep.2013.07.016

11. Bustanji, Y., Al-Masri, I.M., Mohammad, M., Hudaib, M., Tawaha, K., Tarazi, H. & Alkhatib, H.S. 2011. Pancreatic lipase inhibition activity of trilactone terpenes of *Ginkgo biloba*. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 26(4): 453-459. https://doi.org/10.3109/14756366.2010.525509

12. Chia, T.Y., Gan, C.Y., Shafie, M.H., Yap, P.G., Mohd Rodhi, A., Ahmad, A., Murugaiyah, V., Abdulla, M.H. & Johns, E.J. 2023. A comprehensive review on the pancreatic lipase inhibitory peptides: A future anti-obesity strategy. *Electronic Journal of General Medicine*, 20(3): em470. https://doi.org/10.29333/ejgm/12943

13. Cornelio, V.E., Maluf, F.V., Fernandes, J.B., Da Silva, M.F.G.F., Oliva, G., Guido, R.V.C. & Vieira, P.C. 2017. Isolation of tiliroside from *Spiranthera odoratissima* as inhibitor of *Trypanosoma cruzi* glyceraldehyde-3-phosphate dehydrogenase by using bioactivity-guided fractionation. *Journal of the Brazilian Chemical Society*, 28(3): 512-519. https://doi.org/10.21577/0103-5053.20160315

14. de la Garza, A.L., Milagro, F.I., Boque, N., Campión, J. & Martínez, J.A. 2011. Natural inhibitors of pancreatic lipase as new players in obesity treatment. *Planta Medica*, 77(8): 773-785. https://doi.org/10.1055/s-0030-1270924

15. Devi, S. & Kumar, V. 2020. Comprehensive structural analysis of *cis*- and *trans*-tiliroside and quercetrin from *Malvastrum coromandelianum* and their antioxidant activities. *Arabian Journal of Chemistry*, 13: 1720-1730. https://doi.org/10.1016/j.arabjc.2018.01.009

16. EMA (European Medicines Agency). 2022. ICH guideline Q2(R2) on validation of analytical procedures. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q2r2-validation-analytical-procedures-step-2b_en.pdf. (Data accessed: 18.01.2024).

17. Erarslan, Z.B., Colak, R. & Kultur, S. 2021. The preliminary ethnobotanical survey of medicinal plants in Develi (Kayseri/Turkey). *Istanbul Journal of Pharmacy*, 51(2): 263-270. https://doi.org/10.26650/istanbuljpharm.2021.817816

18. Ertas, A., Boga, M., Gazioglu, I., Yesil, Y., Hasimi, N., Ozaslan, C, Yilmaz, H. & Kaplan, M. 2016. Fatty acid, essential oil and phenolic compositions of *Alcea pallida* and *Alcea apterocarpa* with antioxidant, anticholinesterase and antimicrobial activities. *Chiang Mai Journal of Science*, 43(1): 89-99.

19. Fernandes de Oliveira, A.M., Sousa Pinheiro, L., Souto Pereira, C.K., Neves Matias, W., Albuquerque Gomes, R., Souza Chaves, O., Vanderlei de Souza, M.deF., Nóbrega de Almeida, R. & Simões de Assis, T. 2012. Total phenolic content and antioxidant activity of some Malvaceae family species. *Antioxidant*, 1(1): 33-43. https://doi.org/10.3390/antiox1010033

20. Goto, T., Teraminami, A., Lee, J.Y., Ohyama, K., Funakoshi, K., Kim, Y.I., Hirai, S., Uemura, T., Yu, R., Takahashi, N. & Kawada, T. 2012. Tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via activation of adiponectin signaling followed by enhancement of fatty acid oxidation in liver and skeletal muscle in obese-diabetic mice. *The Journal of Nutritional Biochemistry*, 23(7): 768-776. https://doi.org/10.1016/j.jnutbio.2011.04.001

21. Grochowski, D.M., Locatelli, M., Granica, S., Cacciagrano, F. & Tomczyk, M. 2018. A review on the dietary flavonoid tiliroside. *Comprehensive Reviews in Food Science and Food Safety*, 17(5): 1395-1421. https://doi.org/10.1111/1541-4337.12389

22. Hasim, H., Faridah, D.N., Qomaliyah, E.N. & Afandi, F.A. 2023. Pancreatic lipase inhibition activity in lipid absorption using traditional plants: A systematic review and meta-analysis. *Indonesian Journal of Chemistry*, 23(2): 568-582. https://doi.org/10.22146/ijc.76873

23. Irtem-Kartal, D., Eray, N. & Ozturk, F. 2020. Determination of total phenolic and flavonoid contents of some medicinal plants growing in Çaldıran province of Van. *KSU Journal of Agricultural and Nature*, 23(3): 611-616. https://doi.org/10.18016/ksutarimdoga.vi.637632

24. Kirca, A & Arslan, E. 2008. Antioxidant capacity and total phenolic content of selected plants from Turkey. *International Journal of Food Science & Technology*, 43: 2038-2046. https://doi.org/10.1111/j.1365-2621.2008.01818.x

25. Li, X., Tian, Y., Wang, T., Lin, Q., Feng, X., Jiang, Q., Liu, Y. & Chen, D. 2017. Role of the *p*-coumaroyl moiety in the antioxidant and cytoprotective effects of flavonoid glycosides: Comparison of astragalin and tiliroside. *Molecules*, 22(7): 1165. https://doi.org/10.3390/molecules22071165

26. Liu, T.T., Liu, X.T., Chen, Q.X. & Shi, Y. 2020. Lipase Inhibitors for Obesity: A Review. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, 128: 110314. https://doi.org/10.1016/j.biopha.2020.110314

27. Manna, P. & Jain, S.K. 2015. Obesity, oxidative stress, adipose tissue dysfunction, and the associated health risks: Causes and therapeutic strategies. *Metabolic Syndrome and Related Disorders*, 13(10): 423-444. https://doi.org/10.1089/met.2015.0095

28. Nagatomo, A., Nishida, N., Fukuhara, I., Noro, A., Kozai, Y., Sato, H. & Matsuura, Y. 2015. Daily intake of rosehip extract decreases abdominal visceral fat in preobese subjects: a randomized, double-blind, placebo-controlled clinical trial. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 8: 147-156. https://doi.org/10.2147/dmso.s78623

29. Nagatomo, A., Nishida, N., Matsuura, Y. & Shibata, N. 2013. Rosehip extract inhibits lipid accumulation in white adipose tissue by suppressing the expression of peroxisome proliferator-activated receptor gamma. *Preventive Nutrition and Food Science*, 18(2): 85-91. https://doi.org/10.3746/pnf.2013.18.2.085

30. Ninomiya, K., Matsuda, H., Kubo, M., Morikawa, T., Nishida, N. & Yoshikawa, M. 2007. Potent anti-obese principle from *Rosa canina*: structural requirements and mode of action of *trans*-tiliroside. *Bioorganic & Medicinal Chemistry Letters*, 17(11): 3059-3064. https://doi.org/10.1016/j.bmcl.2007.03.051

31. Ndhlala, A.R., Aderogba, M.A., Ncube, B. & Van Staden, J. 2013. Anti-oxidative and cholinesterase inhibitory effects of leaf extracts and their isolated compounds from two closely related *Croton* species. *Molecules*, 18(2): 1916-1932. https://doi.org/10.3390/molecules18021916

32. Panche, A.N., Diwan, A.D. & Chandra, S.R. 2016. Flavonoids: An overview. *Journal of Nutritional Science*, 5: e47. https://doi.org/10.1017/jns.2016.41

33. Polat, R., Cakilcioglu, U. & Satıl, F. 2013. Traditional uses of medicinal plants in Solhan (Bingöl-Turkey). *Journal of Ethnopharmacology*, 148(3): 951-963. https://doi.org/10.1016/j.jep.2013.05.050

34. Sagiroglu, M., Topuz, T., Ceylan, K. & Turna, M. 2013. An ethnobotanical survey from Yahyalı (Kayseri) and Tarsus (Mersin). *SAÜ Fen Edebiyat Dergisi*, 15(2): 13-37.

35. Soltani, S., Koubaa, I., Dhouib, I., Khemakhem, B., Marchand, P. & Allouche, N. 2023. New specific α-glucosidase inhibitor flavonoid from *Thymelaea tartonraira* leaves: Structure elucidation, biological and molecular docking studies. *Chemistry & Biodiversity*, 20(3): e202200944. https://doi.org/10.1002/cbdv.202200944

36. Tetik, F., Civelek, S. & Cakilcioglu, U. 2013. Traditional uses of some medicinal plants in Malatya (Turkey). *Journal of Ethnopharmacology*, 146(1): 331-346. https://doi.org/10.1016/j.jep.2012.12.054

37. Tusevski, O., Kostovska, A., Iloska, A., Trajkovska, L. & Gadzovska-Simic, S. 2014. Phenolic production and antioxidant properties of some Macedonian medicinal plants. *Central European Journal of Biology*, 9(9): 888-900. https://doi.org/10.2478/s11535-014-0322-1

38. Tuzlaci, E. & Eryasar-Aymaz, P. 2001. Turkish folk medicinal plants, Part IV: Gönen (Balikesir). *Fitoterapia*, 72(4): 323-343. https://doi.org/10.1016/s0367-326x(00)00277-x

39. Tuzlaci, E. & Erol, M.K. 1999. Turkish folk medicinal plants. Part II: Eğirdir (Isparta). *Fitoterapia*, 70: 593-610. https://doi.org/10.1016/s0367-326x(99)00074-x

40. Uzunhisarcikli, M.E. & Vural, M. 2012. The taxonomic revision of *Alcea* and *Althaea* (Malvaceae) in Turkey. *Turkish Journal of Botany*, 36: 603-636. https://doi.org/10.3906/bot-1108-11

41. WHO (World Health Organization). 2018. Obesity. https://www.who.int/health-topics/obesity#tab=tab_1 (Date accessed: 27.08.2023).

42. WOA (World Obesity Atlas). 2023. Global overweight and obesity. https://s3-eu-west-1.amazonaws.com/wof-files/World_Obesity_Atlas_2023_Report.pdf (Date accessed: 27.08.2023).