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PHYTOCHEMICAL CHARACTERIZATION AND BIOACTIVITY ASSESSMENT OF *AETHIONEMA SCHISTOSUM* WITH A FOCUS ON ENZYME INHIBITION AND ANTIOXIDANT POTENTIAL

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ABSTRACT. The study examines the antioxidant, antidiabetic, antihyperlipidemic, and antiobesity properties of the 80% methanol extract prepared from the aerial parts of *Aethionema schistosum*, a plant widely distributed in certain regions. The extract was evaluated *in vitro* for its inhibitory effects on *α*-glucosidase (antidiabetic), *α*-amylase (antidiabetic), pancreatic lipase (antiobesity), and pancreatic cholesterol esterase (antihyperlipidemic) enzymes. It exhibited moderate α-glucosidase inhibition (99.15±0.04%) compared to acarbose (56.94±3.88%). The DPPH radical scavenging activity of the extract at a concentration of 2 mg/ml was measured at 80.50±1.23%. This value is considered close to the inhibitory effect of ascorbic acid, which was recorded at 90.60±0.29% at the same concentration. The extract's total phenol (74.73±4.76 mg GAE/g) and flavonoid (42.80±2.25 mg QE/g) contents were measured, and chlorogenic acid was identified as a major compound via HPLC. This is the first study to analyze the phytochemical composition and enzyme inhibitory effects of *A. schistosum*. Further research is needed to isolate its bioactive compounds and assess its therapeutic potential through diverse *in vitro* and *in vivo* models, highlighting its potential in drug discovery efforts.

Keywords: *Antidiabetic, Antioxidant, Aethionema schistosum, Reverse phase-HPLC*

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INTRODUCTION

The Brassicaceae family comprises over 340 genera and approximately 3350 species distributed predominantly in the temperate regions of the Northern Hemisphere. Anatolia is not only the main gene center but also the main diversification center for the genus *Aethionema,* represented by 57 taxa. *A. schistosum* Boiss. & Kotschy, commonly known as Göksun Kayagülü in Turkey, is an endemic perennial herb exhibiting unique morphological and chemical characteristics [1,2].

Studies on the biological activity and phytochemical properties of *Aethionema* species are relatively rare. These studies have focused on the phytochemical analysis of volatile compounds, fatty acids, alkaloids, flavonoids, and phenolic acids of the plant and on the investigation of their antioxidant and antimicrobial activities. On the other hand, literature survey findings also showed that no activity or phytochemical studies have been carried out on *A. schistosum* so far [3].

Diabetes is an endocrine system disease that is defined as an excessive increase in blood glucose levels caused by a combination of hereditary and environmental factors. Diabetes is a chronic metabolic disorder and also has an increased oxidative stress state. Increased free radicals can interact with lipids, proteins, and nucleic acids, leading to loss of membrane integrity and structural or functional changes in proteins. Therefore, internal and external antioxidant pools and supplements are important in diabetes. Coronary artery disease is the most common cause of death in diabetic patients, and hyperlipidemia, which manifests itself with hypertriglyceridemia and low high-density lipoprotein cholesterol levels, is very common, especially in type 2 diabetics. Considering that experts estimate that this disease will affect 693 million adults by 2045, the discovery of new molecules for its treatment is very important. In order to discover new drug molecules from medicinal plants, firstly, folk remedies and secondly, randomly selected plants are used in research [4,5].

In this study, the antidiabetic, antiobesity, and antihyperlipidemic effects of the extract prepared with 80% methanol from the aerial parts of *A. schistosum* were investigated. For this purpose, *α*-glucosidase, *α*-amylase, pancreatic lipase, and pancreatic cholesterol esterase enzymes were used. The antioxidant activity of the methanol extract was evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, ferric reducing power, and metal chelating capacity methods. In addition, the total flavonoid and total phenol contents of the extract were measured by UV spectroscopy, and the Reverse phase-HPLC technique was used to define the phenolic compounds.

RESULTS AND DISCUSSION

The *A. schistosum* MeOH extract exhibited the highest antioxidant activity by effectively scavenging the DPPH radical, as assessed by three different methods. Activity of the extract (80.50±1.23%) was comparable to that of the reference compound, ascorbic acid (90.60±0.29%), at an equivalent concentration (2 mg/ml). The ability of the MeOH extract to reduce ferric ions at a concentration of 2 mg/ml (1.56±0.03) was weaker than that of the reference compound quercetin (3.65±0.11), which was also tested at the same concentration. Table 1 presents the results of ferric reducing power, metal chelating capacity, and DPPH radical scavenging activity.

The ferric-reducing power of MeOH extract at 2 mg/ml concentration (1.56±0.03) was found to be weak compared to the reference compound quercetin (3.65±0.11) also at 2 mg/ml concentration. Ferric reducing power, metal chelating capacity and DPPH radical scavenging activity results are given in Table 1.

Table 1. Metal binding capacity, DPPH radical scavenging, and ferric-reducing power activity results of *A. schistosum* MeOH extract

Sample/ References	Concentration (mg/ml)	DPPH radical scavenging activity % ± SD	Metal chelating capacity % ± SD	Ferric reducing power Absorbance ± SD
MeOH extract	0.01	79.79±0.01$^{***\,a}$	-	0.42±0.01^{***c}
	1	80.14±0.35^{***a}	-	0.82±0.03^{***c}
	2	80.50±1.23^{***a}	-	1.56±0.03^{***c}
^{a}**AA/** ^{b}**EDTA/** ^{c}**QE**	0.01	89.71±0.21^{***a}	99.73±0.91$^{***\,b}$	1.91±0.13^{***c}
	1	90.35±0.20^{***a}	100.00±0.03^{***b}	3.14±0.04^{***c}
	2	90.60±0.29^{***a}	100.00±0.02^{***b}	3.65±0.11^{***c}

-: No activity, SD: Standard Deviation, *p<0.05, **p<0.01, ***p<0.001 AA: ^{a}Ascorbic acid, ^{b}EDTA: Ethylenediamine tetraacetic acid, ^{c}QE: Quercetin

In order to evaluate the antidiabetic effect, the effects of the MeOH extract against *α*-glucosidase and *α*-amylase enzymes were investigated. The inhibition values for these two enzymes were calculated as 56.94±3.88% and 40.55±3.65%, respectively. It was determined that the MeOH extract was ineffective on pancreatic lipase enzyme. Inhibition of pancreatic cholesterol esterase enzyme was calculated as 24.36±3.64% for 200 μg/ml concentration. All percent inhibition results obtained were found to be statistically significant. Enzyme inhibition results are given in Table 2.

Table 2. Inhibitory activities of *A. schistosum* MeOH extract on *α*-glucosidase, *α*-amylase, pancreatic lipase, and pancreatic cholesterol esterase enzymes

Sample/ References	200 µg/mL inhibition % ± SD (IC_{50}: µg/mL ± SD)			
	***α*-Amylase**	***α*-Glucosidase**	**Pancreatic lipase**	**Pancreatic cholesterol esterase**
MeOH extract	40.55±3.65^{***a} (IC_{50}:> 200)	56.94±3.88^{***a} IC_{50}: 155.43±2.50)	- (IC_{50}:-)	24.36±3.64^{***c} (IC_{50}:> 200)
AKA[a]/ ORL[b]/SIM[c]	91.62±1.33^{***a} (IC_{50}: 15.38±1.81)	99.15±0.04^{***a} (IC_{50}: 0.03±0.01)	74.93±3.71^{***b} (IC_{50}: 0.96±0.82)	66.33±2.12^{***c} (IC_{50}: 81.71±1.36)

-: No activity, SD: Standard Deviation, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ AKA: [a]Acarbose, [b]ORL: Orlistat, [c]SIM: Simvastatin

In order to examine the phytochemical profile of the MeOH extract, total phenol and flavonoid contents were determined. The total phenol and total flavonoid contents of the extract were found to be 74.73±4.76 GAE mg/g extract and 42.80±2.25 QE mg/g extract, respectively. In addition, qualitative and quantitative analyses of chlorogenic acid, hesperidin, rutin, and quercetin 3-*O*-glucoside were performed by the Reverse phase-HPLC (Table 3). Chromatograms of rutin, quercetin 3-*O*-glucoside, chlorogenic acid, hesperidin, and the extract are given in Figures 1-3.

Table 3. Amounts of chlorogenic acid, quercetin 3-*O*-glucoside, rutin, and hesperidin in *A. schistosum* MeOH extract

Compounds	Rt (Min.)	g/ 100 g dry extract	Calibration curve	Linearity	LOD (ppm)	LOQ (ppm)
Rutin	30.181	0.100±0.002	y=95.462x +27.260	r^2 =0.9973	0.036	0.119
Chlorogenic acid	15.533	1.420±0.001	y=24.523x +9.432	r^2 =0.9981	0.059	0.176
Quercetin 3-*O*-glucoside	30.812	0.610±0.003	y=66.434x -95.636	r^2 =0.9993	0.011	0.041
Hesperidin	31.123	0.020±0.003	y=36.112x -6.254	r^2 =0.9998	0.021	0.063

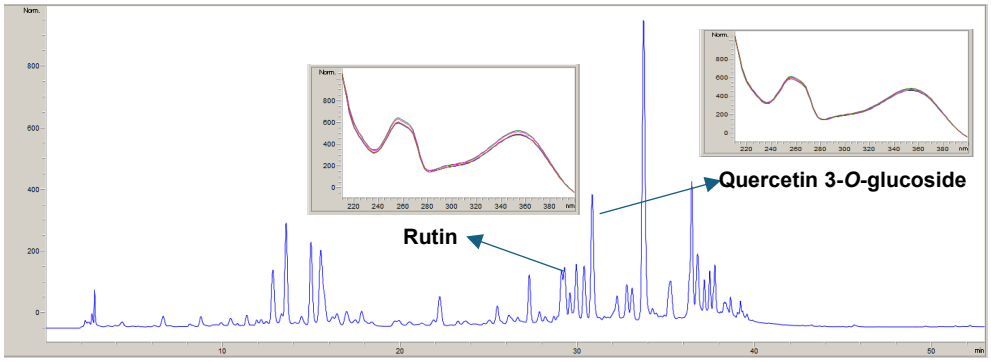

Figure 1. Chromatogram of *A. schistosum* MeOH extract at 260 nm

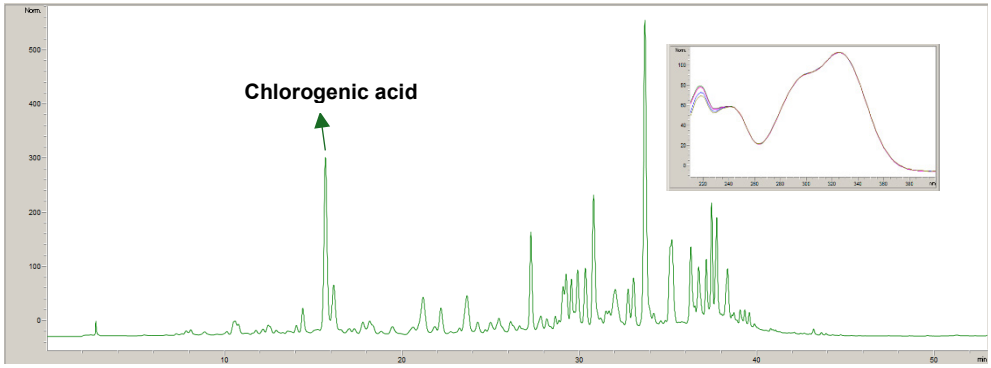

Figure 2. Chromatogram of *A. schistosum* MeOH extract at 320 nm

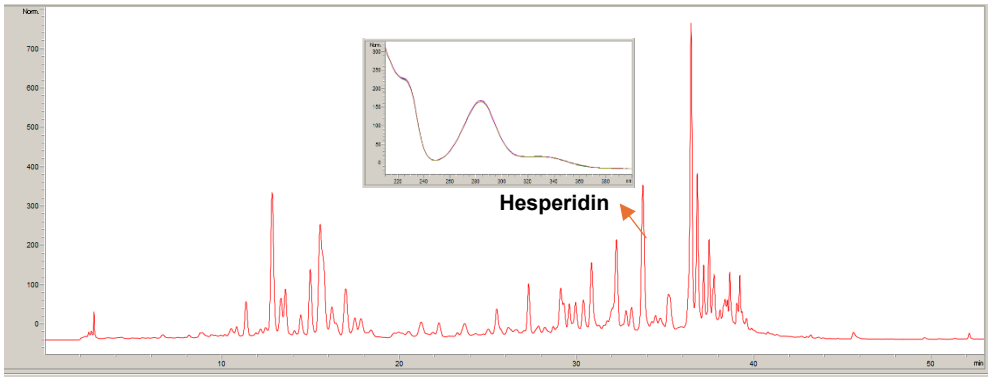

Figure 3. Chromatogram of *A. schistosum* MeOH extract at 280 nm

It was determined that the MeOH extract contained high amounts of chlorogenic acid (1.420±0.001 g/100 g dry extract) and low amounts of hesperidin (0.020 ± 0.003 g/100 g dry extract). Among the phenolic compounds and flavonoids analyzed by HPLC, the highest amount of chlorogenic acid and the lowest amount of hesperidin were determined in the extract. The *Aethionema* genus is a taxon that is widely found in Turkey but has not been studied much. To date, scientific studies based on antioxidant activity studies and isolation and qualitative and quantitative determination of some phytochemical compounds have been conducted on these species belonging to this taxon. Scientific study findings on *Aethionema* species other than *A. schistosum*, which is our plant species, are presented below.

Goffman et al. (1999) reported that *A. grandiflora* Boiss. & Hohen. contained 63.60%, 10.9%, and 11.80% linolenic, oleic, and linoleic acid, respectively, by the GC-MS technique [6].

In a study conducted by Molan et al. in 2012, the antioxidant activities and phenolic contents of 14 plants traditionally used in Northern Iraq, including *A. grandiflorum*, were evaluated. Total phenolic contents, iron-reducing powers, and DPPH radical scavenging effects of the aqueous and ethanolic extracts of the plants were investigated. The total phenolic content of *A. grandiflorum* was determined as 32.7±0.13 and 33.20±0.05 mg GAE/100 ml in aqueous and ethanolic extracts, respectively. Ferric-reducing power was calculated as 12.80±0.06 and 13.60±0.03 in aqueous and ethanolic extracts, respectively. DPPH radical scavenging effect was found to be 22.70±1.1 and 34.03±0.90 in aqueous and ethanolic extracts, respectively [7].

In a study conducted by Duran et al. (2015), the total phenolic contents and antioxidant activities of thirty different plants, including *A. oppositifolium* (Pers.) Hedge and *A. dumanii* Vural & Adıgüzel, were investigated. The plants were extracted in 90% methanol, 9% water, and 1% acetic acid mixtures. The total phenolic content of the extracts was determined by the Folin-Ciocalteu method. The antioxidant activities of the plants were determined by the DPPH radical scavenging activity method. The total phenolic contents of *A. oppositifolium* and *A. dumanii* were determined as 3.337 and 3.267 (mg GAE/100 ml), respectively. Again, the antioxidant activities of the plants were calculated as 81.158% and 78.941%, respectively [8].

Aliyazicioglu et al. (2017) investigated the volatile components, phenolic composition, and antioxidant properties of *A. diastrophis* Bunge and its antibacterial and antifungal activities. Qualitative and quantitative analyses of protocatechuic aldehyde (29.4%), chlorogenic acid (14.3%), and benzoic acid (56.3%) were carried out in the methanol extract, and it was found that the DPPH radical scavenging activity of methanol and aqueous extracts was weaker than butylhydroxytoluene, and the cupric-reducing power of the

aqueous extract (508.00±2.02 µmol Trolox/g extract) was higher than that of the methanol extract (160.00±1.15 µmol Trolox/ g extract). The volatile component mixture obtained from the plant by SPME method was found to be rich in α-humulene and viridiflorene [9].

Demirpolat (2022) identified the essential oil and fatty acid compositions of the aerial parts of *A. sancakense* Yıld. & Kılıç using the GC/GC-MS technique and determined the presence and amount of linoleic acid (23.1%), α-humulene (19.8%), camphene (13.9%), and heptanal (9.7%) [9, 10].

A study by Oboh et al. (2014) found that rutin (diluted to 500 µl) inhibited *α*-amylase (IC_{50} = 0.043 µM) and *α*-glucosidase (IC_{50} = 0.037 µM) activities more potently than quercetin. In this study, the researchers demonstrated the inhibitory effect of quercetin (diluted to 500 µl) and rutin on *α*-amylase and *α*-glucosidase activity *in vitro*. They also demonstrated that this effect has synergistic inhibition abilities, suggesting that a combination of food sources rich in these flavonoids could be very effective in the management/ prevention of Type 2 diabetes [11].

Zheng et al. (2020) reported that chlorogenic acid (0.1-2.0 mg/ml), a phenolic acid commonly found in potatoes, honeysuckle, and other plants, showed a significant inhibitory effect on α-amylase [12].

Panda et al. (2007) investigated the potential of quercetin-3-*O*-glucoside isolated from *Annona squamosa* leaves to regulate alloxan-induced hyperglycemia and lipid peroxidation (LPO) in rats. An increase in serum glucose concentration and a decrease in insulin levels were observed in animals with alloxan-induced diabetes. Following administration of 15 mg/kg quercetin-3-*O*-glucoside daily for 10 days, the animals' blood glucose levels decreased, insulin levels increased, and hepatic glucose-6-phosphatase activities were simultaneously inhibited. These findings indicate that quercetin-3-*O*-glucoside is effective in diabetes mellitus and has the potential to reduce tissue LPO levels [13].

In a study conducted by Aja et al., in which the binding affinities of bisphenol A (BPA) and hesperidin to fibroblast growth factor 21, *α*-amylase, and *α*-glucosidase enzymes were evaluated by the molecular docking method, it was proven that hesperidin (-5.80, -9.60, and -9.60 kcal., respectively) binds to these proteins with a greater affinity than BPA (-4.40, -7.20, and -7.10 kcal.). As a result, it was predicted that hesperidin could be a promising natural compound for metabolic and endocrine disorders [14].

Our study identified and quantified the presence of four compounds in the MeOH extract: rutin, chlorogenic acid, quercetin-3-*O*-glucoside, and hesperidin. Based on the above-mentioned literature, it was considered that rutin, chlorogenic acid, quercetin-3-*O*-glucoside, and hesperidin may contribute to the glucosidase inhibitory effect of *A. schistosum* MeOH extract.

CONCLUSIONS

In conclusion, *A. schistosum* MeOH extract was found to have a relatively moderate effect on *α*-glucosidase enzymes at a concentration of 200 µg/ml. This suggests that this plant may have a therapeutic or supportive effect in diseases associated with these enzymes. However, it is not possible to reach this conclusion directly from these experiments alone. Therefore, these results can be further justified by *in vivo* studies. The aim of our study is to contribute to the realization of this by shedding light on future studies. Since the number of studies of this type is quite limited, it is thought that our study will help other studies to be conducted on this subject. As can be seen, there are no studies evaluating the effects of other *Aethionema* species against these enzymes, which play an important role in metabolic diseases. In this study, both the antioxidant activity and *in vitro* enzyme inhibitory effect potential of *A. schistosum* were investigated for the first time. In this study, the antidiabetic, antihyperlipidemic, and antiobesity effects of *A. schistosum*, a plant commonly grown in Anatolia, were investigated by *in vitro* methods. However, they found that the methanol extract of the aerial part of the plant did not significantly inhibit other enzymes except the α-glucosidase enzyme. In terms of phytochemical studies, it is the first study conducted on the species. As a result, it is recommended that future studies be designed as projects in which various *in vitro* and *in vivo* experimental models will be used and different enzyme activities will be examined in order to better determine the antidiabetic properties of the plant. Additionally, to utilize *Aethionema* species, which are widely distributed in our country and typically regarded as ornamental plants, for medical purposes, activity screening and isolation studies of bioactive compounds should be conducted using *in vitro* enzyme systems.

EXPERIMENTAL SECTION

Plant material

A. schistosum was collected in June 2023 from Ankara, Turkey. The collected plant was identified by Prof. Dr. Osman Tugay (Selçuk University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Konya, Turkey). The specimens of the plant are preserved in the Herbarium of Gazi University Faculty of Pharmacy (Herbarium Number: GUE 3885). The aerial parts of the plant were selected for use. These parts were left to dry in a shaded area at room temperature. The dried plant material was ground into a powder using a mechanical grinder.

Chemicals used

All solvents and chemicals used in the activity evaluation studies, enzyme inhibition assays, and High-Performance Liquid Chromatography (HPLC) methods were of high purity. These chemicals were obtained from Sigma-Aldrich.

Extraction

The powdered aerial parts of *A. schistosum* (10 g) were extracted with 80% methanol (200 ml) at room temperature for 24 hours. A mechanical stirrer (RW20, IKA Janke Kunkel Labortechnik, IKA®-Werke GmbH & Co. KG, Germany) was used during the extraction process. The extraction procedure was repeated three times, and each time, the extracts were filtered using 0.45 µm filter papers. The methanol (MeOH) extract obtained from each extraction was combined and concentrated to dryness under reduced pressure at 45 °C using a rotary evaporator (Heidolph, Germany). (MeOH extract yield: 26.14% w/w dry plant material).

Antioxidant activity assays

DPPH radical scavenging activity

To evaluate the DPPH radical scavenging activity of the extract, a 1 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) solution was added to the extracts (0.01, 1, and 2 mg/ml). The mixture was then incubated for 30 minutes. Absorbance was measured at 520 nm using an ELISA microplate reader. Ascorbic acid (AA) was used as the reference compound [15].

Ferric reducing power

The extracts (0.01, 1, and 2 mg/ml) and the reference compound QE were mixed with 0.1 mol/L sodium phosphate buffer at pH 7.2. Subsequently, a 1% (w/v) $K_3Fe(CN)_6$ solution was added, and the mixture was incubated at 37°C in an incubator. After the incubation, a 10% trichloroacetic acid solution was added to the mixture. The absorbance values were measured at 700 nm using an ELISA microplate reader. Following the first measurement, a 0.1% (w/v) $FeCl_3$ solution was added, and a second absorbance measurement was performed. The difference between the two absorbance values was then calculated. The experiments were conducted in triplicate [16].

Metal chelating capacity

A 2 mM $FeCl_2$ solution was added to the extracts (0.01, 1, 2 mg/ml) and incubated at room temperature for 5 minutes. Subsequently, a 5 mM

ferrozine solution was added, and the mixture was allowed to stand at room temperature for an additional 10 minutes. The absorbance of the extract and the reference compound (Ethylenediaminetetraacetic acid (EDTA)) was measured at 562 nm using an ELISA microplate reader. The experiments were performed in triplicate [17].

Enzyme inhibitory activity tests

α-Glucosidase enzyme inhibition activity

In this study, *α*-glucosidase type IV enzyme (from Bacillus stearothermophilus) (EC 232-604-7 Sigma Co., St. Louis, USA) was used. The enzyme was dissolved in 0.5 M phosphate buffer solution at pH 6.5. *p*-Nitrophenyl-*α*-D-glucopyranoside (PNG) was used as the substrate, which was dissolved in phosphate buffer and adjusted to a concentration of 20 mM. The 80% MeOH extract was prepared at concentrations of 0.01, 1, and 2 mg/ml. The enzyme solution and extract mixture were pre-incubated at 37°C for 15 minutes in a microplate. Subsequently, 20 mM PNG substrate was added to the wells, and the mixture was incubated again at 37°C for 35 minutes. Acarbose (Bayer, Turkey) was used as the reference compound. Absorbance values were measured at 405 nm using an ELISA microplate reader. The experiment was performed in triplicate [18].

α-Amylase enzyme inhibition activity

In this study, *α*-amylase type I-A (EC 3.2.1.1, Sigma) enzyme obtained from porcine pancreas was used. The enzyme was dissolved in sodium phosphate buffer (pH 6.9). Potato starch (2.5% w/v) prepared in a phosphate buffer solution was used as the substrate. The 80% MeOH extract was prepared at concentrations of 0.01, 1, and 2 mg/ml. 3,5-dinitrosalicylic acid (DNS) solution was used as the color reagent. The DNS solution was prepared using 96 mM DNS, 2 M NaOH, and 5.31 M sodium potassium tartrate. Acarbose was used as the reference compound. The *α*-amylase enzyme and sodium phosphate buffer (20 mM NaH_2PO_4 and 6.7 mM NaCl) (pH 6.9) were added to the samples, followed by incubation at room temperature for 5 minutes. The substrate was then added to the mixture, and it was incubated again at 37°C for 15 minutes. Subsequently, DNS was added, and the mixture was incubated at 80°C for 40 minutes in an oven. Absorbance values were measured at 540 nm using an ELISA microplate reader. The amount of maltose produced was found using the standard maltose calibration graph ($y=0.7615x-0.1246$ and $r^2=0.9839$) [19].

Pancreatic lipase enzyme inhibition activity

In this study, the pancreatic lipase type II enzyme (from porcine pancreas) was used. The enzyme buffer solution (pH 6.8) was prepared using 1 mM EDTA and 10 mM 4-morpholinepropanesulfonic acid. The extract was prepared at concentrations of 0.01, 1, and 2 mg/ml. Tris buffer (100 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.0) was added to the extract and the reference compound orlistat, followed by pre-incubation at 37°C for 15 minutes. Subsequently, 5 mM 4-nitrophenyl butyrate was added to the wells, and the mixture was incubated at 37°C for 30 minutes. Absorbance values were measured at 405 nm using an ELISA microplate reader [20].

Pancreatic cholesterol esterase enzyme inhibition activity

In this study, the porcine pancreatic cholesterol esterase enzyme was utilized. The enzyme buffer solution (pH 7) was prepared using 100 mM NaCl and 100 mM. 80% MeOH extract was prepared at concentrations of 0.01, 1, and 2 mg/ml, and 50 µL of phosphate buffer was added to the extract. Following this step, taurocholic acid (12 mM) and 5 mM *p*-NPB (*p*-nitrophenyl butyrate) substrate were added. After incubation at 25°C for 5 minutes, porcine pancreatic cholesterol esterase enzyme was added to the mixture at a concentration of 0.1 µg/ml. Absorbance values were determined kinetically at 405 nm over 15 minutes using an ELISA microplate reader. Simvastatin was used as the reference compound [21].

Chemical composition of the MeOH extract

Total phenolic content

The Folin-Ciocalteu reagent (10% w/v) was added to the extract (1 mg/ml). Following this, a 7.5% (w/v) sodium carbonate solution was added, and the mixture was incubated in the dark at room temperature for 30 minutes. After the incubation, the absorbance value was measured at 735 nm using an ELISA microplate reader (SpectraMax i3x, Molecular Devices, USA). The total phenolic content was calculated as gallic acid equivalents (GAE) in mg/g of extract. The calibration equation was determined to be $y=6.6511x–0.025$ and $r^2=0.9998$ [22].

Total flavonoid content

Aluminum chloride, sodium acetate, and ethanol solutions were added to the MeOH extract (1 mg/ml), followed by incubation at room temperature for 30 minutes. Absorbance values were measured at 415 nm using an ELISA microplate reader. The total flavonoid content was expressed as quercetin equivalents (QE) in mg/g of extract. The calibration curve equation was found as $y=1.8346x–0.004$ and $r^2=0.9988$ [23].

Reverse-Phase HPLC

The HPLC system used in this study was an HP Agilent 1260 Series LC System equipped with an ACE 5 C18 column (5 µm, 150 mm x 4.6 mm). The column temperature was maintained at 25°C throughout the analysis. For the qualitative and quantitative determination of phenolic compounds and flavonoids in the infusion, standard mixtures of the following compounds were used. Phenolic compound mixture: gallic acid, protocatechic acid, chlorogenic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, ellagic acid, caffeic acid, trans-cinnamic acid, rosmarinic acid, epicatechin, catechin. Flavonoid mixture: umbelliferone, rutin, naringenin, hesperidin, quercetin-3-*O*-glucoside, apigenin-7-*O*-glucoside, myrcetin, quercetin, luteolin, apigenin. Standard compounds were sourced from Sigma-Aldrich. A gradient elution system was applied using a mobile phase consisting of 5% solvent A (acetonitrile: water: formic acid, 50:50:0.5) and 95% solvent D (water: formic acid, 100:0.5). The total analysis time was set to 55 minutes, and 20 µL of the sample was injected. Analyses were performed using a DAD detector at five wavelengths: 220, 260, 280, 320, and 366 nm. The extract was prepared at a concentration of 10 mg/ml using a 25% acetonitrile solution. A membrane filter with a pore size of 0.22 µm was used for filtration. Calibration curves were prepared for rutin, quercetin-3-O-glucoside, chlorogenic acid, and hesperidin. Standard solutions of these compounds were prepared by diluting their stock solutions in 25% acetonitrile-water solution to achieve concentrations of 1, 10, 20, 50, and 100 mg/L. Calibration curves were constructed by plotting the mg/L values (x) against the peak areas (y) [3].

Statistical analysis

All analyses were performed in triplicate. All values were calculated as mean ± standard deviation (SD). Linear regression analyses and calculations were conducted using Microsoft Excel, while IC_{50} values were statistically evaluated using GraphPad ANOVA. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

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