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Research paper

Phytochemical profile by LC-MS/MS analysis and evaluation of antioxidant, antidiabetic, anti-Alzheimer, and anticancer activity of *Onobrychis argyrea* leaf extracts

Esma Yeniçeri [a], Ahmet Altay [b,*], Ekrem Koksal [b], Sevgi Altın [a], Parham Taslimi [c], Mustafa Abdullah Yılmaz [e], Oğuz Cakir [e], Abbas Tarhan [e], Ali Kandemir [d]

[a] *Institute of Science and Technology, Erzincan Binali Yıldırım University, 24030, Erzincan, Turkey*
[b] *Department of Chemistry, Faculty of Arts and Science, Erzincan Binali Yıldırım University, 24100, Erzincan, Turkey*
[c] *Department of Biotechnology, Faculty of Science, Bartin University, 74100, Bartın, Turkey*
[d] *Department of Biology, Faculty of Arts and Science, Erzincan Binali Yıldırım University, 24100, Erzincan, Turkey*
[e] *Science and Technology Research and Application Center, Dicle University, 21280, Diyarbakir, Turkey*

A R T I C L E I N F O

Keywords:
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A B S T R A C T

Introduction: Studies on different species of *Onobrychis*, a member of the Fabaceae family, have revealed a number of biological activities with potential applications in alternative medicine. The phytochemical content of the plant *Onobrychis argyrea* subsp. *argyrea* and some of the important biological activities linked to its metabolism are quite limited. Therefore, this study was the first to determine the content of secondary metabolites in this species and to elucidate important biological activities related to metabolism.

Methods: LC-MS/MS was used for the quantitative analysis of bioactive compounds in the aerial part of *O. argyrea* extracts. The antioxidant properties of the extracts were assessed based on their ability to scavenge DPPH radicals and reduce iron ions. The anti-diabetic effect of the extracts was tested by the inhibition of α-amylase and α-glycosidase enzymes, and the anti-Alzheimer's ability was tested by the inhibition of the enzymes AChE and BChE. The cytotoxic effects of the extracts were tested on four different cancer lines, namely A-549, HT-29, MCF-7 and MDA-MB 453 using the XTT assay. The mechanism of the anti-cancer effect was determined by means of flow cytometry analysis on HT-29 cells.

Results: Twenty-nine phytochemicals were identified via the LC-MS/MS analysis in the plant extracts. The most abundant phytochemicals in the extracts were quinic acid, isoquercitrin, epicatechin, and routine, respectively. Antioxidant analysis showed that among all extracts, methanol extract (ME) was most effective in scavenging DPPH (IC_{50}: 23.93 ± 0.96 μg/mL) and in reducing iron ions (53.24 ± 2.14 mgTEAC/g). Enzyme activity studies demonstrated that ME is a potent inhibitor of α-glycosidase (IC_{50}: 15.06±0.64 μg/mL), AChE (IC_{50}: 24.75±0.76 μg/mL), and BChE (IC_{50}: 3.36±0.14 μg/mL). XTT assay results disclosed the strongest anti-proliferative activity of ME against the human colorectal adenocarcinoma HT-29 cell line with an IC_{50} value of 33.35±0.92 μg/mL. Flow cytometry analysis revealed that ME caused mitochondrial membrane damage in HT-29 cells (**$p<0.01$) and subsequently induced apoptosis (**$p<0.01$) by activating the caspase pathway (**$p<0.001$).

Conclusion: O. argyrea, rich in bioactive compounds, showed remarkable antioxidant, antidiabetic, Alzheimer's, and anticancer properties *in vitro*, demonstrating that this plant contains secondary metabolites that may be potential drug candidates for the treatment of metabolic diseases.

Abbreviations: AChE, Acetylcholinesterase; BChE, Butyrylcholinesterase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EAE, Ethyl acetate extract; IC_{50}, Half-maximal inhibitory concentration; LC-MS/MS, Liquid Chromatography-Mass Spectrometry; ME, Methanol extract; TEAC, Trolox equivalent antioxidant capacity; WE, water extract; XTT, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide.

https://orcid.org/0000-0002-6860-9758https://orcid.org/0000-0001-8120-8900https://orcid.org/0000-0003-0853-566Xhttps://orcid.org/0000-0002-0820-912Xhttps://orcid.org/0000-0002-3171-0633https://orcid.org/0000-0002-4090-7227https://orcid.org/0000-0002-8006-2054https://orcid.org/0000-0001-5196-3892https://orcid.org/0000-0003-1902-9631

* Corresponding Author. Dr. Ahmet Altay, Erzincan Binali Yıldırım University, Faculty of Arts and Science, Department of Chemistry, 24100, Erzincan, Turkey, Tel: +90 (0446) 24 30 32

E-mail address: aaltay2013@gmail.com (A. Altay).

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1. Introduction

The *Fabaceae* family is the second largest family worldwide after the *Asteraceae* family.

The family is not only widely used in traditional medicine, but is also commercially important, providing protein and micronutrients for health and subsistence [1]. The genus *Onobrychis* is made up of about 170 species worldwide and includes perennial to annual herbs or shrubs. [2]. Studies of these species have reported that they have a variety of biological functions. For example, *O. gracilis* has been used in traditional medicine to treat the common cold and influenza. [3]. *O. hypargyrea* has been shown to have antioxidant activity and also inhibitory potential against cholinesterase, tyrosinase, α-amylase, and α-glycosidase enzymes [1]. A study on *O. oxyodonta* has shown that it has anti-tumour activity [4]. *O. viciifolia*, a medicinal plant used in Africa, has been reported to have anti-parasitic activity [5]. *O. argyrea*, a species belonging to the Fabaceae family, belongs to the Iranian-Turkish flora and is widely distributed in Turkey's Terrestrial Anatolia, where it is found in steppes and dry slopes [6]. This plant is also widely used as a fodder plant to increase the productivity of calcareous and arid soils [7].

Certain subspecies of *O. argyrea* have been shown to inhibit enzymes associated with metabolic diseases such as diabetes and Alzheimer's disease, and these effects have been attributed to the abundance of phenolic compounds in their structures [8]. They have also been reported to have antioxidant properties that help prevent reactive oxygen species (ROS) that cause or contribute to many diseases [8].

Type 2 diabetes (T2D), which usually occurs in middle-aged people, is a condition in which blood sugar levels are high because the pancreas does not produce enough insulin or does not use it properly. T2D is also associated with other metabolic abnormalities, such as abdominal obesity, high blood pressure and dyslipidaemia, which contribute to very high levels of cardiovascular morbidity and mortality. [9]. Inhibition of some enzymes that are able to hydrolyse polysaccharide sugars and convert them into monosaccharides is one of the defined therapeutic targets for the treatment of diabetes. [10]. Since the enzyme α-glycosidase plays an important role in the hydrolysis of oligosaccharides and disaccharides into simple sugars, α-glycosidase enzyme inhibitors (AGIs) can prevent the absorption of simple sugars in the GI tract, thereby prolonging the time of carbohydrate digestion and preventing the development of T2D [11,12].

Alzheimer's disease (AD) is a neurodegenerative disorder characterised by progressive memory loss and cognitive impairment. [11]. AD involves oxidative stress, inflammation and a lack of acetylcholine in specific parts of the brain [13]. Acetylcholinesterase (AChE), a serine protease that is able to hydrolyse the production of ACh, thereby reducing ACh and disrupting neuronal signalling, contributes to the pathogenesis of AD [11,14]. Because acetylcholinesterase (AChE) has been shown to play a major role in the development or progression of Alzheimer's disease, several AChE inhibitors (AChEI), including rivastigmine, tacrine, and donepezil, have been used to treat this neurodegenerative disease [14]. On the other side, these drugs have several disadvantages, including lower activity, lower selectivity and higher toxicity, so new drugs with low toxicity, high selectivity and high activity are needed to treat AD [15]. Another AChE-dependent serine hydrolase is butyrylcholinesterase (BChE), which is the best substrate for BChE. Although the function of BChE is not fully understood, selective BChE inhibitors (BChEIs) have been defined to increase ACh levels and reduce abnormal amyloid production in Alzheimer's disease. [14].

Cancer is one of the leading causes of death in the world and is the second most common cause of death after cardiovascular disease [16]. Conventional cancer treatments, chemotherapy and radiotherapy, are known to be expensive and cause many side effects, from minor ones like vomiting, alopecia, diarrhoea, and constipation to major ones like neurological, cardiac, and renal toxicity [17–19]. All of these side effects reduce the quality of life and discourage patients from adhering to medication protocols, which can lead to progression of the cancer and complications [18,19]. Additionally, there is therefore a need to discover alternative anti-cancer drugs that are hopefully more potent, selective and less toxic than those currently used. Natural sources, including medicinal plants, account for 60% of the drugs currently used to treat cancer. [20].

The aim of this study was to determine the phytochemical content of different extracts obtained from the leaf parts of O. argyrea by LC-MS/MS analysis and to evaluate their antioxidative, anti-diabetic, anti-Alzheimer and anticarcinogenic properties *in vitro*.

2. Methods

2.1. Chemicals

Folin-ciocalteus, sodium carbonate, aluminum nitrate, ammoniumacetate, 2 2-diphenyl-2- picrylhydrazyl (DPPH), potassium ferrocyanide, trichloroacetic acid, acetylcholinesterase, α-glycosidase, butyrylcholinesterase, 5,5′-dithio-bis(2-nitro-benzoic) acid (DTNB), p-NPG, 5-Fluorouracil (5-FU), hexane, ethyl acetate and, methanol were purchased from Sigma Aldrich (St. Louis, MO, USA). 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H tetrazolium-5-carboxanilide (XTT), Annexin V-FITC/PI double-staining, Mitochondrial membrane potential (MMP), and Multi-caspase activity kits were purchased from Biological Industries (BI, USA).

2.2. Instruments

Rotary evaporator (RE 100 Bibby, Stone Staffordshire, UK), freeze drier (FD-1000, Eyela, Japan), Ultrahigh performance liquid chromatography (UHPLC, Shimadzu-Nexera, SIL-30AC, Japan), Mass spectrometric detection (Shimadzu LCMS-8040, Japan), microplate reader (BioTek, USA), Carbondioxide incubator (NUVE, Turkey), Bio-safety cabinet (NUVE, Turkey), Muse™ Cell Analyzer (Darmstadt, Germany).

2.3. Plant material

The plant species was collected from the same region of Hürrempalangası, Yalnızbağ, Erzincan province during the flowering period on 13.06.2020. The plants were dried in the dark under air-flow, and the leaves were crushed and homogenized to a fine powder. The plant material was identified by Dr. Ali Kandemir (Kandemir 11149) and kept at the herbarium of Erzincan Binali Yıldırım University, Erzincan, Turkey.

2.4. Preparation of the plant extracts

After the plant sample was collected, the aerial parts of the plant (1 kg) to be studied were cut into small pieces and powdered with the help of a mixer. The sequential extraction method was used for the extraction procedures. Firstly, 200 g of the powdered sample was macerated with 400 mL of hexane at room temperature and stirred for 12 h to obtain defatted plant powders. This procedure was performed two times. Afterward, to obtain ethyl acetate extract, 200 g of defatted powder was treated with 400 mL of ethyl acetate solution at room temperature for 12 h two times. The same procedure was carried out for methanol extract (ME) and water extract (WE). All the extracts were filtered over Whatman paper (No.1) and the filtrates were concentrated using a rotary evaporator at 60 °C and a freeze drier. Three different time periods were used for the extraction process, and the fractionation with solvents was repeated two times in each of the procedures. The obtained extracts were stored at -20°C in a dark glass bottle to be used for LC–MS/MS analyses and biological activity studies.

2.5. LC-MS/MS analysis

The individual phytochemical composition of *O. argyrea* was

determined over EAE (ethyl acetate extract), ME (methanol extract), and WE (water extract). The final concentration of each extract was adjusted to 1 mg/mL with methanol before LC–MS/MS analysis. The test samples were kept at a constant degree (15°C) throughout the experiment. Analysis of individual phytochemicals in all extracts was performed over 56 standard phytochemicals. The mass spectrometer and chromatograph conditions, total ion chromatogram, and analytical validation parameters belonging to the standard phenolic compounds were shown in Fig. S1 and Table S1.

2.6. Antioxidant activity

2.6.1. Total phenolic and flavonoid contents

Total phenolic contents (TPC) of the extracts from *O. argyrea* were determined according to Folin Ciocalteu assay with slight modifications [21]. The TPC results were expressed as mg GAE/gram extract. Each extract (0.1 mL) was mixed with 4.6 mL of distilled water (dH_2O). Sodium carbonate (Na_2CO_3) solution (0.3 mL, 2%) and Folin-Ciocalteus reagent (0.1 mL) were added to this mixture and mixed again. The mixture was incubated for 2 h at room temperature (RT). After the incubation period, the absorbance was measured at 760 nm.

The total flavonoid contents (TFC) of each extract were measured by aluminum chloride colorimetric assay with some modifications [22,23]. The TFC results were expressed as mg QE/gram extract. Accordingly, the plant extract (0.1 mL) was added to the test tube including 4.8 mL of methanol, 0.1 mL of aluminum nitrate (10%), and 0.1 mL of ammonium acetate solution (1M). The mixture was incubated for 45 min at RT. After the incubation, the absorbance was measured at a wavelength of 415 nm. Each experiment was performed in triplicate and results were expressed with standard deviations ±SD.

2.6.2. DPPH radical scavenging assay

DPPH radical scavenging capacity of the samples was performed using the Blois method with minor modifications [21,24]. Accordingly, the plant extracts (150 µL) at varying concentrations (5-100 µg/µL) were mixed with 50 µL of DPPH solution (0.1 mM) and incubated for 30 min at RT. Afterward, the absorbance was measured at 517 nm. Trolox was used as a positive control. The results were expressed with the IC_{50} values meaning that the concentration of the extracts scavenging 50% of DPPH radical. Each experiment was performed in triplicate and results were expressed with standard deviations ±SD.

2.6.3. Ferric ion reducing antioxidant power assay

Ferric ions reducing antioxidant power (FRAP) of each extract was carried out to the procedure described by Oyaizu with some modifications [17,21]. Accordingly, each plant extract (37.5 µL) prepared at different concentrations (5-100 µg/µL) was added into a 96-well plate including sodium phosphate buffer (62.5 µL, 0.2 M, pH: 6.6) and potassium ferrocyanide solution (62.5 µL, 1%) and incubated at 50°C for 20 min. After the incubation, the mixture was acidified with trichloroacetic acid (62.5 µL, 10%). Then, $FeCl_3$ solution (25 µL, 0.1%) was added and the absorbances were recorded at 700 nm. The result of each extract was expressed as mg TE/gram extract. Trolox was used as a positive control. Each experiment was performed in triplicate and results were expressed with standard deviations ±SD.

2.7. Enzyme activity studies

2.7.1. Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) assays

AChE and BChE inhibitory effects of the extracts were determined according to the Ellman [25] and Daryadel [26] methods, respectively. Briefly, the extracts at different concentrations (10–150 µg/mL) prepared in 100 µL of Tris/HCl buffer (1.0 M, pH = 8.0) were added into the test tubes including 50 µL (5.32×10^{-3} EU) of both enzyme solutions. After incubate for at 20°C for 15 min, 50 µL of 5,5′-dithio-bis (2-nitro-benzoic) acid (DTNB) (0.5 mM) and acetylthiocholine iodide (AChI /butrylcholine iodide (BChI) were added to the mixtures and the absorbances of the mixtures were recorded at 412 nm for 5 min. The results were expressed with their IC_{50} values showing the concentration of the samples required to inhibit the enzyme by 50%. The reference tacrine was used as a positive control. Each experiment was performed in triplicate and results were expressed with standard deviations ±SD.

2.7.2. α-Glycosidase assay

α-Glycosidase inhibition activity studies were carried out using the method described by Tao [27]. For this, the extracts (5 µL) prepared in ethanol were added to the test tube containing 75 µL of phosphate buffer (0.15 U/mL, pH 7.4) and 20 µL of enzyme solution and incubated at 35°C for 10 min. After the incubation, p-nitrophenyl glucopyranoside (p-NPG) (20 µL) in phosphate buffer (5 mM, pH= 7.4) was added and incubated further at 35°C. The absorbances were measured at 405 nm, spectrophotometrically. The results were expressed as IC_{50} showing the concentration of the samples to inhibit the enzyme by 50%. Acarbose was used as a positive control. Each experiment was performed in triplicate and results were expressed with standard deviations ±SD.

2.7.3. α-Amylase assay

α-Amylase inhibition activity was performed according to iodine/potassium iodide (IKI) method [28]. Accordingly, 30 µL of KH_2PO_4, 20 µL test solution and, 30 µL α-amylase solution were mixed in a test tube and pre-incubated at 37°C for 10 min. Afterward, 50 µL of substrate was added to each well and the reaction mixture was further incubated at 37°C for 10 min. At the end of the incubation period, the reaction was stopped with 25 µL of 10% HCl. After addition of 100 µL of IKI solution, the absorbance of the test tubes was measured spectrophotometrically at 630 nm.

2.8. Anticancer activity

2.8.1. Cell culture studies

The human breast cancer (MCF-7 and MDA-MB-453) cell lines, lung cancer (A-549) cell line, and colon cancer (HT-29) cell line were purchased from the ATCC (American Type Culture Collection). The cultures were incubated at 37°C in a humidified CO_2 (5%) incubator (NUVE, Turkey). Studies were applied in a Bio-safety cabinet (NUVE, Turkey).

2.8.2. Cytotoxicity assay

The cytotoxic activity studies were performed against human breast cancer (MCF-7 and MDA-MB-453), lung cancer (A-549), and, colon cancer (HT-29) cell lines using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H tetrazolium-5-carboxanilide (XTT) assay [29–31]. Accordingly, the cells (5×10^3 cells/well) were seeded into 96-well culture plates and incubated overnight in the 5% CO_2 incubator (NUVE, Turkey). Then, the cells were exposed to the different concentrations (10-250 µg/mL) of ME and WE for 48 h. After the incubation period, the previous medium was aspirated, followed by the addition of 100 µL of fresh medium and 50 µL of XTT substrate and then incubation for 5 h in the 5% CO_2 incubator. Following the incubation, the absorbance showing the cellular metabolic activity of the cells was measured at 470 nm using a microplate reader (BioTek, USA). The cytotoxicity results of the extracts were expressed with IC_{50} values representing the half-maximal inhibitory concentrations of the extracts. 5-Fluorouracil (5-FU) was used as positive control. Each experiment was performed in triplicate and the results were expressed with their standard deviations ±SD.

2.8.3. Apoptosis assay

The apoptotic effect of the ME was examined on HT-29 cells using Annexin V-FITC/PI double-staining assay. Determination of the apoptotic effect of the ME was determined by obeying to the manufacturer's instructions (Muse® AnnexinV/Dead cell assay kit, MCH100105)

[30,32]. Accordingly, the cells (3×10^5 cells/well) were planted into a 6-well culture plate and exposed to the IC_{50} value of the ME for 48 h. After incubation, the collected cells in 1 mL of complete medium including fetal bovine serum (FBS, 1%). Afterward, the cell suspension (100 µL) was mixed with FITC AnnexinV/ propidium iodide and vortexed for 3-5 seconds. After incubation for 20 min at room temperature and monitored by Muse™ Cell Analyzer. The flow cytometry results were represented with dot blot graphs. Untreated cells were used as control group cells Each experiment was performed triplicate.

2.8.4. Mitochondrial membrane potential (MMP) assay

The impact of the ME over MMP disruption in HT-29 cells was determined according to the manufacturer's instructions (MCH100110, Merck Millipore) [33]. For this, the cells (1×10^5 cells/well) were seeded into a culture plate and incubated overnight in a 5% CO_2 incubator. Then, the cells were treated with the IC_{50} concentration of the ME for 48 h. After the exact incubation time, the cells were collected and washed with 1X assay buffer and centrifuged at 300g for 5 min. The cells (100 µL) were stained with MitoPotential solution (95 µL) and incubated for 20 min in a CO_2 incubator at 37°C. After incubation, 7-amino actinomycin D (7-AAD) was added to the test tube and incubated for a further 5 min at RM. Finally, the cells were analyzed using the Muse™ Cell Analyzer. Untreated cells were used as control group cells. Each experiment was performed in triplicate.

2.8.5. Multi-caspase activity assay

The effect of the ME on multi-caspase activity in HT-29 cells was measured by using Muse Cell Analyzer (MCH100109) [33,34]. Accordingly, the cells (1×10^5 cells/well) were seeded into a culture plate and incubated overnight in a 5% CO_2 incubator. Upon the cells taking morphological shapes, they were exposed to the IC_{50} value of ME for 48 h. Then, the cells were collected in 1X caspase buffer and 50 µL of cell suspension was mixed with. 10 µL of Muse Multicaspase Reagent and incubated for 30 min in a 5% CO_2 incubator at 37°C. After incubation, Muse Caspase 7-AAD working solution (2 µL) was added to the test tube and incubated for 5 min at RT. The changes in caspase activity in the cells were analyzed by of Muse™ Cell Analyzer. Untreated cells were used as control group cells. Each experiment was performed in triplicate.

2.9. Statistical analysis

Statistical analyses for the biological activity studies, One-Way ANOVA, and Student's t-test were performed via a statistical program of GraphPad Prism 6 (GraphPad, La Jolla, CA) Software 7.0). All analyses were carried out in triplicate ($n = 3$) and the data was presented as mean ± SD. $P<0.05$ was taken as the minimum level of significance.

3. Results

3.1. LC-MS/MS analysis

The individual phytochemicals of the plant extracts (EAE, ME, and WE) from O. argyrea were identified and quantified by LC–MS/MS analyses, and the chromatograms belonging to the extracts were shown in Fig. S2. Depending on the polarity index of the solvent, differences were observed in the amount and type of the secondary metabolites present in the extracts. The results displayed that twenty-nine different phytochemicals were determined in the plant extracts screened against 56 standard phytochemicals (Table 1). Twenty-eight types of metabolites were identified in ME, twenty-four types of phytochemicals in WE, and twenty types of phytochemicals in EAE. The highest amount of phytochemicals in ME were quinic acid (30.022 mg/g extract), isoquercitrin (11.583 mg/g extract), epicatechin (10.292 mg/g extract), rutin (9.161 mg/g extract), hesperidin (5.797 mg/g extract), and astragalin (1.031 mg/g extract), respectively (Table 1). The most abundant phytochemicals in EAE were isoquercitrin (3.362 mg/g extract), rutin (2.975 mg/g extract), and hesperidin (2.551 mg/g extract), respectively (Table 1). The highest phytochemicals in WE were quinic acid (20.238 mg/g extract), protocatechin (5.888 mg/g extract), isoquercitrin (5.557 mg/g extract), epicatechin (5.447 mg/g extract), rutin (4.116 mg/g extract), hesperidin (3.167 mg/g extract), miquelianin (2.335 mg/g extract) and aconitic acid (1.305 mg/g extract), respectively. The other phytochemicals in the extracts were detected below 1 mg/g extract (Table 1).

Table 1
Phytochemicals of *O. argyrea* extracts by LC-MS/MS analysis.

Analyte	ME	(mg/g extract) EAE	WE
Quinic acid	30.022	0.854	20.238
Fumaric acid	N.D.	N.D.	N.D.
Aconitic acid	0.172	N.D.	1.305
Gallic acid	0.09	0.022	0.571
Epigallacatechin	2.18	N.D.	N.D.
Protocatechuic acid	0.347	0.253	5.888
Catechin	N.D.	N.D.	0,174
Gentisic acid	N.D.	N.D.	N.D.
Chlorogenic acid	0.027	0.018	0.031
Protocatechuic aldehyde	0.01	0.038	0.009
Tannic acid	0.016	N.D.	N.D.
Epigallocatechin gallate	N.D.	N.D.	N.D.
1,5-dicaffeoylquinic acid	N.D.	N.D.	N.D.
4-OH Benzoic acid	N.D.	N.D.	N.D.
Epicatechin	10.292	0.696	5.447
Vanilic acid	N.D.	N.D.	N.D.
Caffeic acid	0.014	0.012	0.027
Syringic acid	N.D.	N.D.	N.D.
Vanillin	N.D.	N.D.	N.D.
Syringic aldehyde	N.D.	N.D.	N.D.
Daidzin	N.D.	N.D.	N.D.
Epicatechin gallate	N.D.	N.D.	N.D.
Piceid	N.D.	N.D.	N.D.
p-Coumaric acid	0.114	0.094	N.D.
Ferulic acid-D3-IS	N.A.	N.A.	N.A.
Ferulic acid	N.D.	N.D.	N.D.
Sinapic acid	N.D.	N.D.	N.D.
Coumarin	N.D.	N.D.	N.D.
Salicylic acid	0.029	0.016	0.08
Cyranoside	0.135	0.099	0.013
Miquelianin	0.389	0.218	2.335
Rutin-D3-IS	N.A.	N.A.	N.A.
Rutin	9.161	2.975	4.116
isoquercitrin	11.583	3.362	5.557
Hesperidin	5.797	2.551	3.167
O-Coumaric acid	N.D.	N.D.	N.D.
Genistin	N.D.	N.D.	N.D.
Rosmarinic acid	N.D.	N.D.	N.D.
Ellagic acid	N.D.	N.D.	N.D.
Cosmosiin	0.008	0.014	N.D.
Quercitrin	0.02	N.D.	N.D.
Astragalin	1.031	0.483	0.738
Nicotiflorin	0.122	0.095	0.167
Fisetin	N.D.	N.D.	N.D.
Daidzein	N.D.	N.D.	N.D.
Quercetin-D3-IS	N.A.	N.A.	N.A.
Quercetin	0.235	0.025	1.271
Naringenin	0.013	0.031	0.01
Hesperetin	N.D.	N.D.	N.D.
Luteolin	0.054	0.061	0.015
Genistein	N.D.	N.D.	N.D.
Kaempferol	0.049	0.072	0.062
Apigenin	0.004	0.018	N.D.
Amentoflavone	N.D.	N.D.	N.D.
Chrysin	0.004	0.017	N.D.
Acacetin	0.043	0.114	0.005

ME: Methanol extract
EAE: Ethyl acetate extract
WE: Water extract
N.D.: Not Detected
N.A.: Not Applicable
IS: Internal Standard

3.2. Total phenolic and flavonoid contents

Total phenolic content (TPC) of *O. argyrea* extracts were determined by the Folin-Ciocalteu method and the results were given in Table 2. ME was found to contain the highest level of TPC (92.38±1.05 mg GAE/g extract), followed by WE (73.25±2.02 mg GAE/g extract), and EAE (25.63±1.05 mg GAE/g extract), respectively. The total flavonoid content (TFC) of the extracts was found to be in parallel with the results of the TPC. The highest TFC was observed in the ME (21.72±0.84 mg QE/g extract), followed by EAE (8.86±0.95 mg QE/g extract), and WE (6.78 ±0.60 mg QE/g extract), respectively.

3.3. DPPH radical scavenging and Ferric ions reducing power

The DPPH radical scavenging capacity of *O. argyrea* extracts was investigated, and the results, together with their IC_{50} values, were presented in Table 2. The results showed that the radical scavenging capacity of extracts is highly correlated with their phenolic content. ME disclosed the highest DPPH radical scavenging activity with its lowest IC_{50} value of 23.93±0.96 µg/mL, followed by WE (IC_{50} = 47.52±1.35 µg/mL) and EAE (IC_{50} = 86.90±1.45 µg/mL), respectively. In addition, it was found that the ME had remarkable activity in comparison to Trolox (IC_{50} =12.80±0.54 µg/mL), the standard source of antioxidants.

Ferric ions reducing abilities of the plant extracts were investigated and the results were represented as trolox equivalent (TE) in Table 2. Similar to the radical scavenging results, the most effective Fe^{3+} reducing capacity was achieved by ME (53.24±2.14 mgTE/g extract), followed by EAE (24.64±1.12 mgTE/g extract) and WE (23.44±1.50 mgTE/g extract), respectively.

3.4. Enzyme activity

The inhibition of the α-glycosidase enzyme by various extracts of *O. argyria* was studied and the results compared with acarbose, a standard inhibitor. Considering the IC_{50} values of the samples in Table 3, the strongest inhibition against α-glycosidase was exhibited by ME with the lowest IC_{50} value of 15.06±0.64 µg/mL, followed by EAE (53.31±0.72 µg/mL), acarbose (69.03±0.92 µg/mL), and WE (77.01±1.30 µg/mL), respectively.

The results of the α-amylase inhibition assay also showed a correlation with the results of the α-glycosidase activity assay. ME showed the strongest inhibition of the α-amylase enzyme with the lowest IC_{50} value of 1.24±0.22 mg/mL, followed by WE (IC_{50}=2.46±0.35 mg/mL) and EAE (IC_{50}=3.31±0.52 mg/mL), respectively (Table 3). Acarbose, on the other hand, showed a slightly stronger inhibition of the amylase enzyme with a lower IC_{50} value compared to the extracts.

The inhibitory effects of *O. argyria* extracts on AChE and BChE enzymes were studied and the results were summarized in Table 3. The results showed that the strongest inhibition of AChE was exhibited by ME with the smallest IC_{50} value of 24.75±0.76 µg/mL, followed by WE (IC_{50}: 40.76±0.50 µg/mL), and EAE (IC_{50}: 46.21±1.02 µg/mL), respectively. Furthermore, ME was shown to have a much more potent inhibitory effect compared to tacrine, which has an IC_{50} of 38.50±1.08 µg/mL. With respect to BChE, it was found that all extracts had a more potent inhibitory effect than tacrine, with lower IC_{50} values. The ranking of the inhibitory potency of the test samples on the BChE enzyme was as follows ME (IC_{50}=3.36±0.14 µg/mL)>EAE (IC_{50}=5.41±0.38µg/mL)> WE (IC_{50}=9.01±0.65µg/mL)> Tacrin (IC_{50}=14.74±0.8 µg/mL).

Table 2
Antioxidant activity results of *O. argyrea* extracts.

Samples	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH IC_{50}(µg/mL)	FRAP (mg TE/g)
EAE	25.63±1.82[a]	8.86±0.95[a]	86.9±1.45[d]	24.64±1.12[a]
ME	92.38±1.05[c]	21.72±0.84[c]	23.93±0.96[b]	53.24±2.14[b]
WE	73.25±2.02[b]	6.78±0.60[b]	47.52±1.35[c]	23.44±1.50[a]
Trolox	-	-	12.80±0.54[a]	-

TPC: Total phenolic content
TFC: Total flavonoid content
EAE: Ethyl acetate extract
ME: Methanol extract
WE: Water extract
Data marked with different superscripts within the same column (a-d) shows a significant difference (p< 0.05).

Table 3
α-Glycosidase, α-Amylase, AChE, and BChE inhibitory activities of *O. argyrea* extracts.

Extracts/ Standards	α-Glycosidase IC_{50} (µg/mL)	α-Amylase IC_{50} (mg/mL)	AChE IC_{50} (µg/mL)	BChE IC_{50} (µg/mL)
EAE	53.31 ±0.72 [b]	3.31±0.52 [d]	46.21±1.02 [c]	5.41±0.38 [b]
ME	15.06 ±0.64 [a]	1.24±0.22 [b]	24.75±0.76 [a]	3.36±0.14 [a]
WE	77.01 ±1.30 [d]	2.46±0.35 [c]	40.76±0.50 [b]	9.01±0.65 [c]
Acarbose	69.03 ±0.92 [c]	0.86±0.14 [a]	-	-
Tacrine	-	-	38.50±1.08 [b]	14.74±0.80 [d]

IC_{50} results are expressed as the mean ± SD (n = 3). Mean values followed by different letters in the same column are significantly different (P ≤ 0.05).

3.5. Cytotoxicity

The potential cytotoxic effects of the extracts from *O. argyrea* were investigated on four different cancer lines, namely HT-29 (human colorectal adenocarcinoma), MCF-7 and MDA-MB-453 (human breast carcinoma), and A-549 (human lung cancer). XTT assay results showed that each extract exhibited a dose-dependent cytotoxicity after 48 h, depending on the cell type (Fig. 1). The cytotoxicity of the extracts against HT-29 cells ranged from 1 to 100 µg/mL. This range increased to 250 µg/mL for MCF-7, MDA-MB-453, and A-549 cells.

Upon the ranking of the strength of the cytotoxic effect of the extracts on A-549 cancer cells was examined in Table 4, ME was found to display the strongest antiproliferative effect with the lowest IC_{50} value of 119.48 ±2.02 µg/mL, followed by WE (154.05±1.95 µg/mL), and EAE (191.49 ±2.54 µg/mL), respectively. On HT-29 cells, ME was approximately two times more cytotoxic (IC_{50}: 33.35±0.92µg/mL) than WE (IC_{50}: 61.20 ±1.24µg/mL) and six times more cytotoxic than EAE (IC_{50}: 118.81 ±3.10µg/mL). It was also found to have a cytotoxic potency close to that of 5-FU (IC_{50}: 32.22 ± 1.25 µg/mL), which is used as a standard chemotherapeutic agent. Similarly, the order of the cytotoxic activity of the extracts against MCF-7 cells was as follows: ME (105.32±1.86 µg/mL)> WE (149.54±2.30 µg/mL)> EAE (198.87±3.12 µg/mL). The order of cytotoxic activity of the extracts against MDA-MB-453 cells was ME (145.99±2.14 µg/mL)> EAE (185.19±2.40 µg/mL)> WE (197.36 ±3.06 µg/mL).

3.6. Flow cytometry results

To determine the apoptotic effect of ME in HT-29 cells, the cells were treated with the ME for 48 h and followed by Annexin V-FITC/PI application. The data showed that ME increased the early apoptotic rate in HT-29 cells from 9.5%±1.08 to 23.20%±1.52 (***p<0.001) and the late apoptotic rate from 4%±0.74 to 8%±0.58. (*p<0.05) (Fig. 2).

To determine the effect of ME of *O. argyrea* on mitochondrial membrane damage in HT-29 cells, these cells were exposed to the IC_{50} concentration of ME for 48 h and the results were analyzed by flow cytometry. As shown in Fig. 3, the cell viability decreased from 68.10% ±2.10 to 23.90%±1.46 (**p<0.01), the depolarised-living cell population increased from 7.25%±0.38 to 22.90%±1.20 (**p<0.01) and the depolarised-dead cell population did not change significantly (p>0.05)

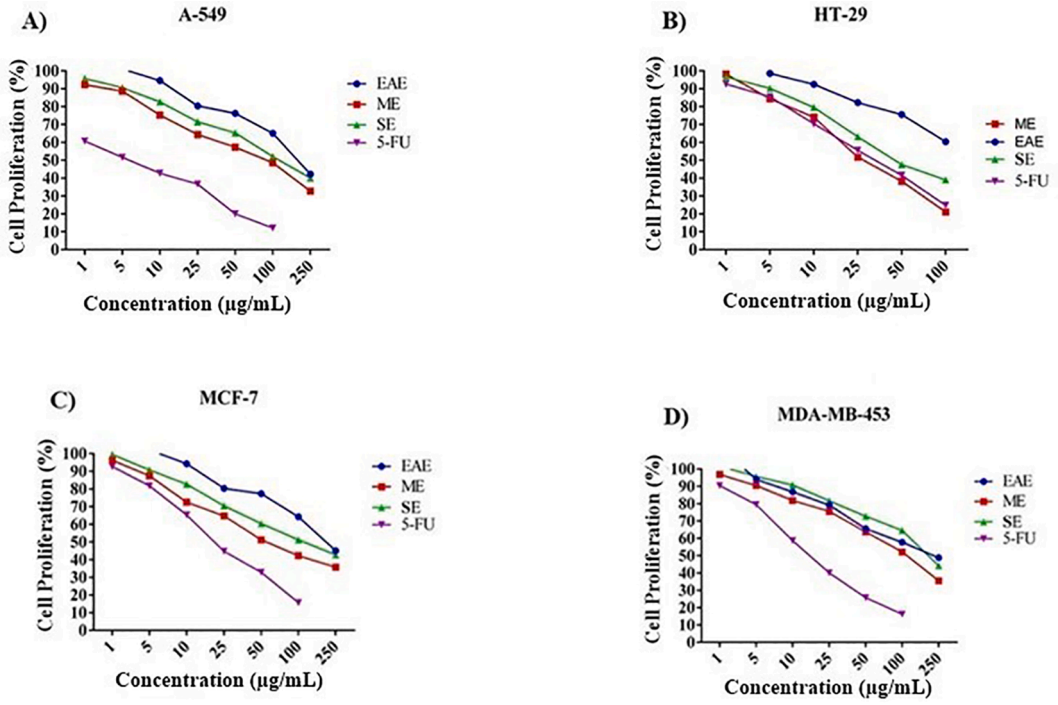


Fig. 1. Dose-dependent cytotoxic effects of *O. argyrea* extracts on A-549 (**A**), HT-29 (**B**), MCF-7 (**C**), and MDA-MB-453 (**D**) cells. Experiments were performed in 3 replicates.

Table 4
Half maximal inhibitory concentrations (IC_{50}: µg/mL) of *O. argyrea* extracts against human breast (MCF-7 and MDA-MB-453), lung (A549), and colon (HT-29) cell lines.

Extracts/Standard Chemotherapeutic	A-549	HT-29	MCF-7	MDA-MB-453
EAE	191.49 ±2.54 ^{d}	118.81 ±3.10 ^{c}	198.87 ±3.12 ^{d}	185.19 ±2.40 ^{c}
ME	119.48 ±2.02 ^{b}	33.35 ±0.92 ^{a}	105.32 ±1.86 ^{b}	145.99 ±2.14 ^{b}
WE	154.05 ±1.95 ^{c}	61.20 ±1.24 ^{b}	149.54 ±2.30 ^{c}	197.36 ±3.06 ^{d}
5-FU	18.90 ±1.24 ^{a}	32.22 ±1.25 ^{a}	23.2 ±0.78^{a}	29.52 ±1.54 ^{a}

Data marked with different superscripts within the same column (a-d) indicates a significant difference ($p< 0.05$).

in the ME-treated HT-29 cancer cells.

To determine whether ME induces the caspase cascade in HT-29 cells after MMP damage, cells were incubated with the IC_{50} value of ME for 48h and analysed by flow cytometry. The results showed that the caspase-positive living-cell population increased from 3.10%±0.24 to 15.25%±1.38 (***$p < 0.001$) and also the caspase-positive dead-cell population increased from 2.00%±0.26 to 8.155%±0.95 (***$p < 0.001$) in the ME-treated HT-29 cells, compared to the control group (Fig. 4).

4. Discussion

For the quantitative analysis of small molecules in food and feed samples, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become the most widely used technique [69]. In the present study, many phenolic acid and flavonoids from *O. argyrea* extracts were determined by LC-MS/MS analyses. When the biological activities of these phytochemicals were investigated, Quinic acid, the major component of the plant extracts, has been reported to have many biological properties, including anti-inflammatory [36], antioxidant [37], anti-hepatitis B [38], antiviral activity, anti-nociceptive [39], anti-diabetic [40], cytotoxic, and apoptotic [41] activities. Isoquercetin, which is found in the second highest amount, was shown to disclose significant biological properties such as antioxidant and anti-inflammatory activities [42], antiviral activity [43], anticancer activity via mitochondrial-dependent apoptosis in SK-Mel-2 melanoma cells and anti-diabetic activity in type II diabetic rats [44,45]. Epicatechin was shown to have considerable biological activities in the treatment of major metabolic diseases such as cancer and diabetes [46], and cardiovascular and neurophysiological treatments [47].

Rutin was shown to have cardioprotective, cytoprotective, neuroprotective, anticarcinogenic, and antioxidant activities [48]. Protocatechuic acid has been known to exhibit antiproliferative, antioxidant, and antiadipogenesis properties [49]. Hesperidin, an important flavonoid, was reported to exhibit cardioprotective, antihyperlipidemic, antidiabetic, and antihypertensive activities [13], antioxidant capacity in biological systems [50], apoptotic effect on human colon cancer cells (SNU-C4) via caspase-3 activation [51]. Miquelianin, quercetin 3-O-glucuronide, has been known to display antioxidant and antidiabetic [52], antiproliferative and anti-inflammatory [53] effects. Quercetin, a flavonoid, has received increasing attention for its antioxidant, antiobesity, anticarcinogenic, antiviral, antibacterial, and anti-inflammatory effects [54–56]. Additionally, the other chemicals found at lower levels in the extracts were also shown to have numerous biological properties [57–59].

In addition to the identification of individual phytochemicals in plant extracts, total phenolic and flavonoid content can also be determined spectrophotometrically using the Foline and Aluminium methods, allowing the phenolic content of medicinal plants to be compared. In literature, TPC of methanol and water extracts from *O argyrea* subsp. *isaurica* was reported as 76.06 and 78.09 mg GAE/g extract, respectively. Also, TFC results of the same extracts were found to be 1.63 and 2.11 mmol TE/g extract, respectively [60]. In another study, different extracts (methanol, ethanol, water, dichloromethane, and n-hexane) were obtained from the flower part of *O. armena* by Soxhlet extraction, and their phenolic and flavonoid contents were

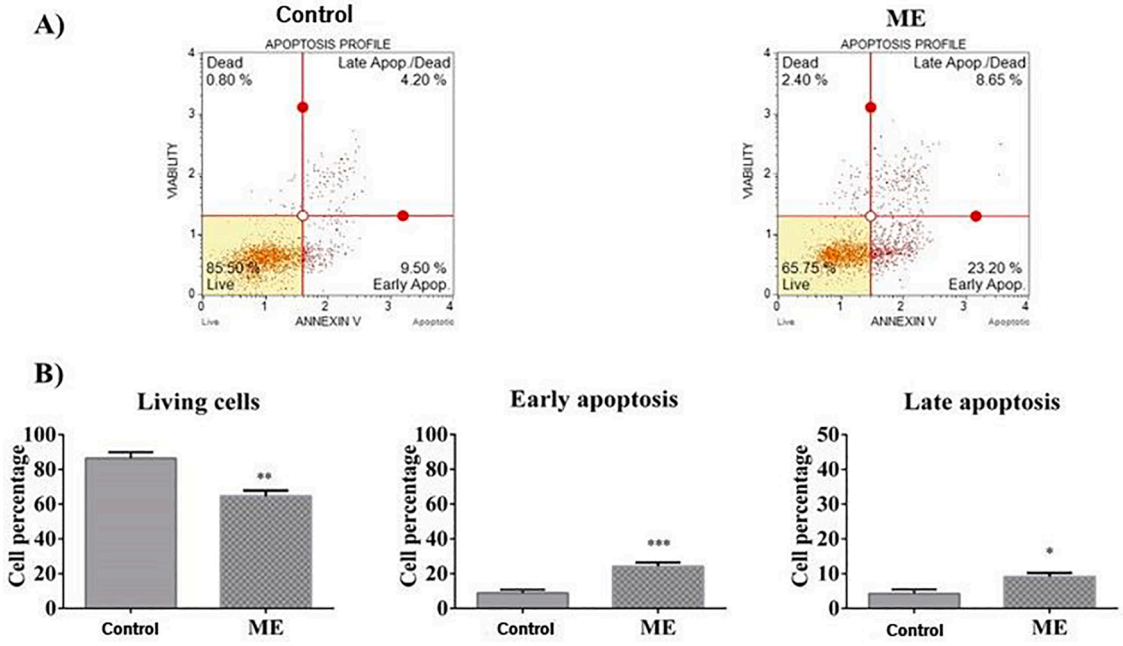


Fig. 2. Apoptotic effect of *O. argyrea* methanol extract on HT-29 cells. A) Flow cytometry dot plot graph. B) Statistical results of viable, early apoptotic, and late apoptotic cell populations. *p < 0.05 (significant), **p < 0.01 (highly significant), ***p < 0.001 (very significant), ****p < 0.0001 (extremely significant).

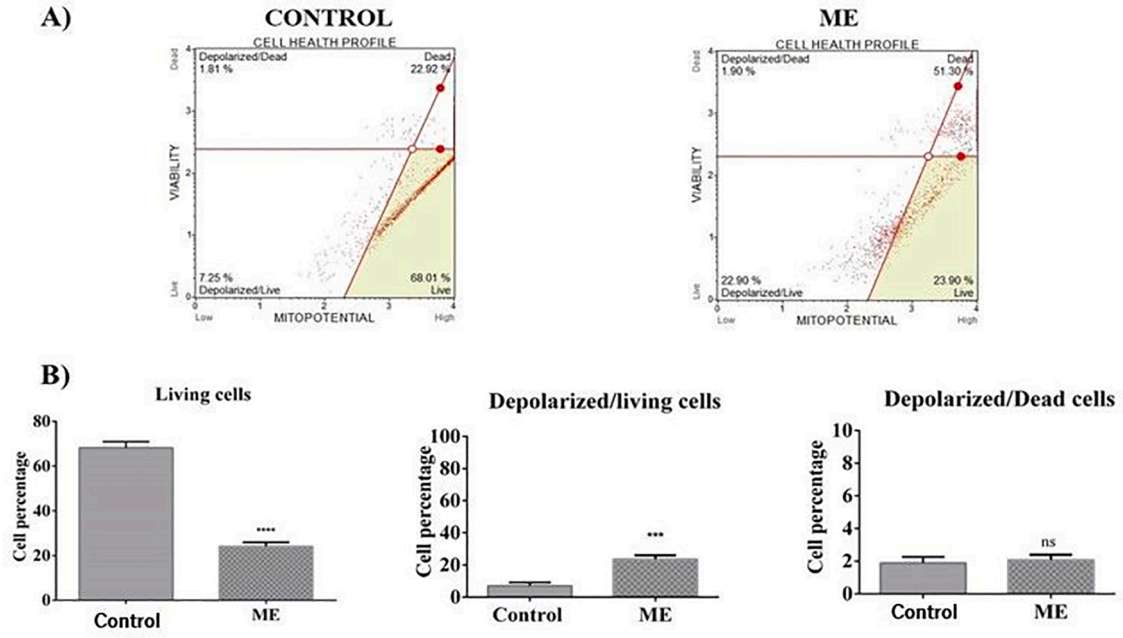


Fig. 3. Effect of *O. argyrea* methanol extract on mitochondrial membrane depolarization in HT-29 cells. A) Flow cytometry dot plot graph. B) Statistical results of viable, depolarized/viable, and depolarized/dead cell populations. Experiments were performed in 3 replicates. *p < 0.05 (significant), **p < 0.01 (highly significant), ***p < 0.001 (very significant), ****p < 0.0001 (extremely significant). ns (no significant, p>0.05).

investigated. The results showed that the highest phenolic and flavonoid contents were found in the methanol extract with 128.23 mg GAE/g extract and 23.92 mg QE/g extract, respectively [61]. Similar results were shown in another study, where it was determined that among different extracts obtained from *O. nitida*, the methanol extract contained the highest amount of phenolic compounds with a value of 101.73 mg GAE/g extract. [62]. These data obtained confirmed the LC-MS/MS results, i.e. the highest phenolic and flavonoid content was found in the methanolic extract. In addition, it showed similar results with the phenolic amounts obtained from studies conducted with different species of the *Onobrychis* plant in the literature.

Many biological activities of medicinal plants have been attributed to the phenolic and/or flavonoid compounds that they contain [35].

Reactive oxygen species (ROS) are a class of highly reactive molecules derived from oxygen metabolism. In addition, ROS can cause extensive damage to cells and tissues during infections and various degenerative diseases such as cardiovascular disease, ageing and neurodegenerative diseases such as Alzheimer's, mutations and cancer [70]. Sophisticated defence mechanisms for radical detoxification are present in mammalian cells. Key metabolic steps include superoxide

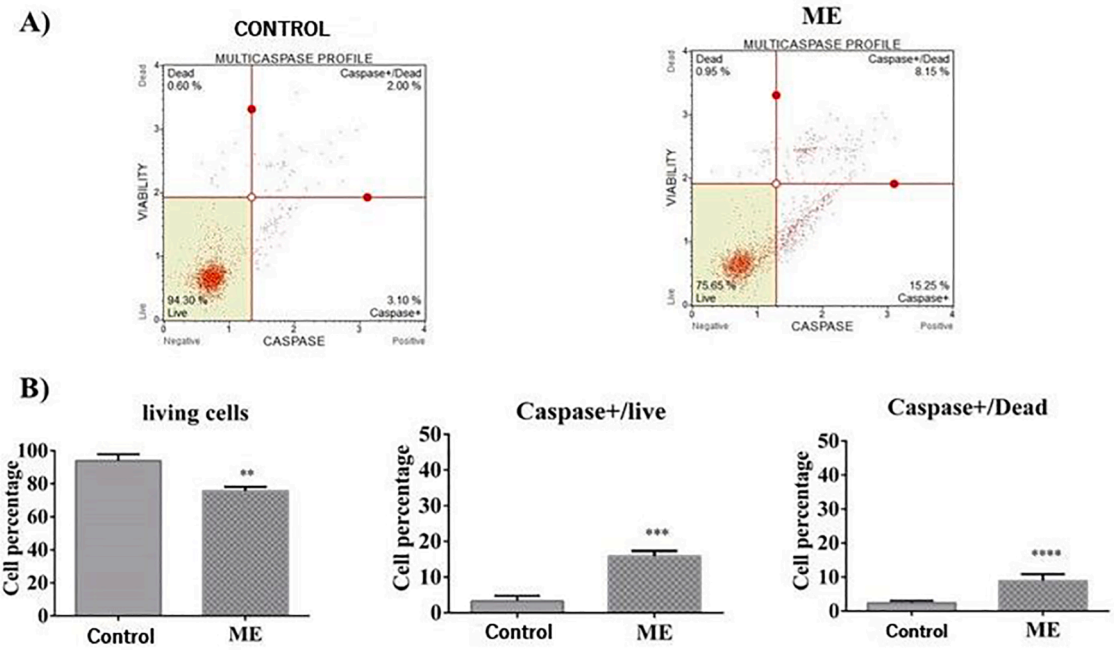


Fig. 4. Effect of *O. argyrea* methanol extract on multi-caspase activity in HT-29 cells. A) Flow cytometry dot plot graph. B) Statistical results of viable, caspase/viable, and caspase/dead cell populations. Experiments were performed in 3 replicates. *p < 0.05 (significant), **p < 0.01 (highly significant), ***p < 0.001 (very significant), ****p < 0.0001 (extremely significant).

dismutase (SOD) catalysing the dismutation of superoxide to hydrogen peroxide and oxygen, and the conversion of H_2O_2 to water and oxygen by catalase (CAT) and glutathione peroxidase (GPX), which destroys toxic peroxides. In addition to antioxidant enzymes, several small molecule antioxidants play an important role in the antioxidant defence system. There are two types of compounds: those produced by the body and those produced by food [71].

DPPH radical scavenging assay is a commonly used method to investigate the antioxidant capacity of a natural or synthetic compound due to their notable roles in removal of free radicals from biological systems [63]. Another essential method that is widely used to determine the antioxidant capacity of the compounds is the FRAP assay, which is based on the reduction of ferric-tripyridyltriazine (Fe^{III}-TPTZ) by antioxidants to ferrous-tripyridyltriazine (Fe^{II}-TPTZ) [64]. Thus, the formation of hydroxyl radicals, which can occur in biological systems as a result of Fenton reactions and cause various metabolic diseases by damaging many macromolecules, was measured by FRAP tests and the removal of these radicals from the environment was determined by the DPPH method. For this reason, these two methods were considered complementary in determining the antioxidant capacity of the extracts.

Studies carried out with different species of *Onobrychis* in the literature showed that the antioxidant powers of these species vary depending on the antioxidant assay, the extraction method, and the part of the plant used. For instance, in a study performed with the methanol extract obtained from the leaf part of *O. argyrea*, the DPPH radical scavenging and FRAP results were reported as 126.51 mg TE/g extract and 200.70 mg TE/g extract, respectively [8]. In another study, radical scavenging and metal-reducing power experiments were performed on the methanol extract of *O. nitida* and the IC_{50} value of DPPH result was 77.29 μg/mL, and FRAP result was 518.87 TE/g extract [62]. Karakoca et al. investigated the methanol extract of *O. armena* and reported the IC_{50} value of DPPH radical scavenging as 669.23 μg/mL [61]. Another study reported that ethylacetate extract was found to have the strongest DPPH radical scavenging activity among the different extracts obtained from *O. crista-galli* (L.) with an IC_{50} value of 17.3 μg/mL [65]. These data confirmed that *O. argyrea* is highly rich in biologically active compounds that prevent or eliminate the formation of radicals in biological systems.

Type 2 diabetes is the most common form of diabetes and is characterised by the body's inability to respond to normal levels of circulating insulin, also known as insulin resistance. Current anti-diabetic therapy is based on synthetic drugs, which often have side effects. For this reason, there is a constant need to develop new and better drugs as alternatives for the management and treatment of the disease. Natural hypoglycaemic compounds may be attractive alternatives to synthetic drugs or adjuncts to currently used treatments. Their major advantage is that they can be taken as part of the daily diet. Recently, more attention has been paid to the study of natural products as potential antidiabetic agents [72].

Due to the fact that excessive activity of α-glycosidase and α-amylase enzymes is strongly associated with type 2 diabetes (T2D), many chemical compounds have been synthesized to inhibit these enzymes. However, as most of these compounds are synthetic and can have many side effects on the metabolism, there has been a shift in recent years towards obtain these compounds from natural sources [10]. Another important metabolic disorder is Alzheimer's disease, in which choline esterases play a pivotal role. Therefore, the suppression or inhibition of these enzymes by external stimuli plays a key role in eliminating or halting this disease [66]. The results from enzyme activity studies showed that the *O. argyrea* plant, particularly its methanol extract, inhibited the enzymes α-glycosidase, AChE, and BChE more than tacrine and acarbose, which were used as standard compounds, and competed with acarbose in inhibiting the enzyme α-amylase. This confirms that the ME of the plant has a very high amount or variety of secondary metabolites in the fight against Alzheimer's and diabetes.

Cancer is one of the most devastating diseases of our time and is a major public health problem as well as a leading cause of death around the world. Great efforts have been made to find more effective drugs with fewer side effects in an attempt to halt the progression of the disease. In particular, natural phenolic compounds have assumed a very important role in cancer prevention because they are the main constituents of fruits and vegetables and are essential components of a healthy diet, as well as other medicinal herbs and plant extracts. They have also shown interesting results in inhibiting the proliferation of several cancer cell lines, showing potential for further investigation as anti-cancer

agents [73].

In this respect, determining cytotoxic effects on cancer cells has been the first step in evaluating natural and/or synthetic anticancer compounds in recent years. [30]. Our results showed that the ME had the most potent cytotoxic effect against all the cancer cell types, and HT-29 cells were the most sensitive to this extract. This could be attributed to the rich phytochemical content of ME, especially quinic acid, isoquercitrin, epicatechin and routine, or their synergistic effects.

A detailed review of the cytotoxicity studies conducted with the extracts from different species of this plant in the literature revealed that the species used in the current study had a much higher cytotoxic potential. For example, in a study, methanol extracts from different species of *Onobrychis* (*O. albiflora, O. ealeeifolia, O. tournefortii,* and *O. arkvrea*) were tested for their cytotoxic activities on colon cancer HTC116 cells and their IC_{50} values were found to have different values between 0.98 and 3.99 mg/mL [7]. Considering these data, it can be thought that the results we obtained in our study strengthen the idea that this plant exhibits very strong cytotoxicity against cancer cells.

Programmed cell death plays an important role in many physiological processes in embryonic development and in adult tissue. Defects in apoptosis regulation contribute to many diseases, including those involving cell accumulation (cancer, restenosis) or cell loss (stroke, heart failure, neurodegeneration, AIDS) [74]. Apoptosis plays an important role in maintaining a balance between cell division and cell death, thus helping to keep the number of cells in a living organism at a constant level [67]. Many chemotherapeutic agents are widely used in the treatment of cancer due to their ability to induce apoptosis. However, it is known that the majority of chemotherapeutic agents have cytotoxic effects and unwanted side effects on healthy cells as well as on cancer cells [19]. For this reason, in addition to synthetic drugs, studies on the anti-cancer effects of crude extracts of medicinal plants or secondary metabolites isolated from them have gained momentum in the last few years [68]. This study investigated whether the mechanism of anticancer action of ME in HT-29 cells is related to apoptosis. Flow cytometry assays revealed that ME specifically drived HT-29 cells to early apoptosis.

In the apoptotic signalling pathways, death receptor- or mitochondrion-dependent apoptosis is initiated by the recruitment and activation of apical caspases. In mitochondrion-dependent apoptosis, an apoptosome containing cytochrome C, Apaf-1 and caspase-9 is formed by the release of cytochrome C into the cytosol. The initiator caspases at the top of the caspase signalling cascade are thought to be caspase-8, caspase-10 and caspase-9. Any disturbance in the mitochondrial membrane causes cytochrome c to be released into the cytosol, where it causes the activation of many caspases, known as the caspase cascade, leading to the activation of caspase-3, directly linked to apoptosis [23]. Our advanced mechanistic anticancer studies performed by flow cytometry analyzes confirmed that ME disrupts the integrity of the mitochondrial membrane in HT-29 cells and subsequently triggers apoptosis in the cells by activating the caspase cascade.

5. Conclusion

This study revealed that the methanol extract obtained from *O. argyrea* plant has significant antioxidant, antidiabetic, anti-Alzheimer's, and anticancer potential with its rich phytochemical content. This opens the door to the isolation of secondary metabolites in the structure of the plant and their subjection to *in vivo* studies.

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CRediT authorship contribution statement

Esma Yeniçeri: Writing – original draft, Investigation. **Ahmet Altay:** Writing – review & editing, Project administration, Methodology. **Ekrem Koksal:** Investigation. **Sevgi Altın:** Investigation. **Parham Taslimi:** Writing – review & editing, Investigation. **Mustafa Abdullah Yılmaz:** Validation, Methodology. **Oğuz Cakir:** Validation. **Abbas Tarhan:** Validation. **Ali Kandemir:** Resources, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The materials and data of this study are available from the corresponding author upon request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.eujim.2024.102337.

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