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Research Article/Araştırma Makalesi

Assessment of in vitro cytotoxicity, anti-Alzheimer, and antidiabetic properties of fenugreek, white mulberry, and nettle leaves

Çemen otu, beyaz dut ve ısırgan otu yapraklarının in vitro sitotoksik, anti-Alzheimer ve antidiabetik özelliklerinin değerlendirilmesi

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ABSTRACT

Leafy plants are known for their rich bioactive profiles and have gained attention for their potential health benefits. This study evaluated the total phenolic content (TPC) using the Folin-Ciocalteu method and antioxidant properties, including ferric reducing antioxidant power (FRAP) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) activities, of ethanolic extracts from fenugreek (FL), white mulberry (WBL), and nettle leaves (NL). It also investigated their inhibitory effects on alpha-amylase, alpha-glucosidase, acetylcholinesterase, and butyrylcholinesterase, and assessed their cytotoxicity on human embryonic kidney cells (HEK-293) and colorectal adenocarcinoma cells (CaCo-2) using MTT assays. The results revealed that the TPC was highest in NL (241.86 mg gallic acid equivalents (GAE) g^{-1} dry weight (DW)), followed by WBL (165.68 mg GAE g^{-1} DW) and FL (72.09 mg GAE g^{-1} DW), with NL also showing the highest FRAP (240.48 µmol Fe^{2+} g^{-1} extract) and ABTS antioxidant activities (19.26 mg trolox equivalents (TE) g^{-1} extract). Moreover, the inhibition of alpha-amylase ranged from 8.85% to 90.39% depending on the extract concentration (62.5–500 µg mL^{-1}), with WBL and NL showing significant inhibitory effects on alpha-glucosidase within the same concentration range. Additionally, NL ethanolic extracts exhibited the highest butyrylcholinesterase inhibitory activity at 38.40% compared to FL (33.87%) and WBL (17.94%) at 2 mg mL^{-1}, while acetylcholinesterase inhibition rates ranged from 23.14% for WBL to 53.35% for NL across all leaf samples. Furthermore, the ethanol extracts from FL, WBL, and NL yielded IC_{50} values of 1159.98, 1235.67, and 972.22 µg mL^{-1}, respectively, on HEK-293 cells, while on CaCo-2 cells, the IC_{50} values were 897.41 µg mL^{-1} for FL, 754.11 µg mL^{-1} for WBL, and 648.80 µg mL^{-1} for NL. These findings underscore the potential of NL, FL, and WBL as valuable natural sources with diverse health benefits and significant therapeutic potential, making them promising candidates for industrial applications as functional ingredients.

Key Words: Fenugreek leaves, mulberry leaves, nettle leaves, cytotoxic activity, enzyme inhibition activity

ÖZ

Yapraklı bitkiler, zengin biyoaktif bileşenler barındırmalarıyla dikkat çeker ve bu bileşiklerin sağlık üzerindeki olumlu etkileri, son yıllarda giderek artan bir ilgiyle

araştırılmalarına yol açmıştır. Bu çalışma, çemen otu (FL), beyaz dut (WBL) ve ısırgan otu yapraklarının (NL) etanol ekstraktlarının toplam fenolik içeriğini (TPC) ve demir indirgeme antioksidan gücü (FRAP) ile 2,2'azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) serbest radikali giderme aktivitelerini içeren antioksidan özelliklerini değerlendirmiştir. Ayrıca, bu ekstraktların alfa-amilaz, alfa-glukozidaz, asetilkolinesteraz ve bütirilkolinesteraz üzerindeki inhibe edici etkileri de araştırılmış ve insan embriyonik böbrek hücreleri (HEK-293) ve kolorektal adenokarsinom (Caco-2) hücre hatlarındaki sitotoksisiteleri değerlendirilmiştir. Sonuçlar, TPC'nin NL'de (241.86 mg GAE g^{-1} DW) en yüksek olduğunu, bunu WBL (165.68 mg GAE g^{-1} DW) ve FL'nin (72.09 mg GAE g^{-1} DW) takip ettiğini ortaya koymuştur. NL ayrıca WBL ve FL'ye kıyasla en yüksek FRAP (240.48 µmol Fe^{2+} g^{-1} ekstrakt) ve ABTS antioksidan aktivitelerini (19.26 mg TE g^{-1} ekstrakt) göstermiştir. Ayrıca, alfa-amilaz inhibisyonu, ekstrakt konsantrasyonuna (62.5–500 µg mL^{-1}) bağlı olarak %8.85 ile %90.39 arasında değişmiş, WBL ve NL aynı konsantrasyon aralığında alfa-glukozidaz üzerinde önemli inhibe edici etkiler göstermiştir. NL etanol ekstraktları, 2 mg mL^{-1} konsantrasyonda FL (%33.87) ve WBL (%17.94) ile karşılaştırıldığında en yüksek bütirilkolinesteraz inhibisyon aktivitesini (%38.40) sergilemiştir. Asetilkolinesteraz inhibisyon oranları ise %23.14 (WBL) - %53.35 (NL) arasında değişmiştir. Ayrıca, FL, WBL ve NL'nin etanol ekstraktlarının IC_{50} değerleri HEK-293 hücrelerinde sırasıyla 1159.98 µg mL^{-1}, 1235.67 µg mL^{-1} ve 972.22 µg mL^{-1} iken, Caco-2 hücrelerinde IC_{50} değerleri FL için 897.41 µg mL^{-1}, WBL için 754.11 µg mL^{-1} ve NL için 648.80 µg mL^{-1} olarak hesaplanmıştır. Bu bulgular, FL, NL ve WBL'nin hem potansiyel sağlık faydalarını hem de dikkate değer terapötik potansiyelini vurgulayarak onları endüstriyel uygulamalar için fonksiyonel bileşen olarak değerlendirilebilecek umut verici doğal kaynaklar haline getirmektedir.

Anahtar Kelimeler: Çemen otu yaprakları, dut yaprakları, ısırgan otu yaprakları, sitotoksik aktivite, enzim inhibitör aktivitesi

Introduction

Herbal remedies, derived from the diverse resources of nature, form a core part of traditional medicine systems in various cultures. For centuries, these natural treatments have been essential in managing health and disease, serving as vital tools for everyday wellness and specialized therapeutic needs across numerous cultures (van Wyk, 2020). Among these valuable remedies are well-known plants such as fenugreek, nettle, and mulberry. For instance, fenugreek, scientifically known as *Trigonella foenum-graecum* L. and belonging to the family Leguminosae, is a medicinal plant traditionally used for both its seeds and leaves. These components are valued not only for their culinary applications but also as crucial ingredients in various traditional medicinal formulations (Verma et al., 2010). Fenugreek has been traditionally used to treat disorders such as high cholesterol, diabetes, gastrointestinal ailments, and wound inflammation. Fenugreek is also acclaimed for its potential anticancer properties, thanks to its advantageous active chemical constituents. Its mechanism of action closely resembles that of various anticancer medications (Alsemari et al., 2014). It contains a variety of active compounds, including saponins (such as protodioscin, dioscin, diosgenin and yamogenin), flavonoids (quercetin, maackiaian, medicarpin, aglycones kaempferol, quercetin, tricin, and narin genin, afroside, luteolin, vitexin, quercitrin, and 7, 4-dimethoxy flavanones), alkaloids (trigonelline), and lignans (secoisolariciresinol) (Nagulapalli et al., 2017; Shadab et al., 2024). Moreover, the genus *Morus* (mulberry) comprises over 150 species, with *Morus alba* L. (white mulberry) standing out as the most significant. Renowned for its powerful therapeutic effects and minimal toxicity, *M. alba* has been extensively utilized in traditional Chinese medicine. Its broad spectrum of health benefits includes antioxidant, antibacterial, antihypertensive, anti-hyperglycemic, neuroprotective, skin tonic, and anti-hyperlipidemic properties (Gryn-Rynko et al., 2016; Zafar et al., 2013). It is abundant in (poly)phenolic compounds, including flavonoids such as quercetin-3-O-glucoside, quercetin-3-O-(6-malonyl)-glucoside, and kaempferol-3-O-(6-malonyl)-glucoside, alongside phenolic acids like caffeic acid and caffeoylquinic acids (Sánchez-Salcedo et al., 2015), as well as flavonols such as rutin, isoquercitrin, and astragalin (Mahboubi, 2019). Furthermore, nettle (*Urtica dioica* L.) is a perennial wild herb from the Urticaceae family, commonly found in temperate regions of Europe, Asia, and America. It typically thrives in untamed

fields, along roadways, on slopes, and in open forested areas, and is distinguished by its light or dark green leaves. Additionally, it demonstrates hypolipidemic, anti-inflammatory, antiviral, antiulcer, antimicrobial, and antioxidant effects, thanks to its active constituents, which include polyphenols, essential amino acids, vitamins (K, B, and C), fatty acids, dietary fibers, carotenes, and terpenes (Kutlu et al., 2020; Kutlu, 2021). It includes hydroxybenzoic acid, gallic acid, quinic acid, syringic acid, vanillic acid, protocatechuic acid, gentisic acid, caffeic acid, and coumaric acid, along with numerous derivatives of quinic and caffeic acids. Additionally, flavonoids such as quercetin, apigenin, catechin, and pelargonidin were detected in both glycosidic and non-glycosidic forms. Furthermore, stinging nettle leaves were found to contain lutein, violaxanthin, neoxanthin, β-carotene, and lycopene (Garcìa et al., 2021; Kregiel et al., 2018). Previous studies have reported on the cytotoxic, anticancer, and enzyme inhibitory activities of fenugreek leaves (Alsemari et al., 2014; Aylanc et al., 2020; Ganeshpurkar et al., 2013; Prithiksha et al., 2022; Ullah et al., 2016; Verma et al., 2010), white mulberry leaves (Chen et al., 2023; Panyatip et al., 2022; Qin et al., 2015), and nettle leaves (Asgharian, 2017; Sharma et al., 2023) were reported in previous studies. However, no research has been concurrently assessed and compared these properties among various plant leaves within a single study. For this reason, the current study sought to (i) evaluate the total phenolic content and antioxidant properties, specifically FRAP and ABTS activities, (ii) investigate enzyme inhibitory activities targeting alpha-amylase, alpha-glucosidase, acetylcholinesterase, and butyrylcholinesterase, and (iii) assess the cytotoxic effects on the human embryonic kidney HEK-293 and human colorectal adenocarcinoma Caco-2 cells, using ethanolic extracts from fenugreek, white mulberry, and nettle leaves.

Materials and Methods

Materials

The plants, specifically *Trigonella foenum graecum* L., *Morus alba* L., and *Urtica dioica* L., were collected in May 2023 from the region located at 39° 20′ 14″ N latitude and 36° 2′ 29″ E longitude in İnkışla, Gemerek district, Sivas province, Türkiye (Figure 1). After collection, the plants were cleaned to remove soil and dust, then dried in a Memmert UF-110 oven (Germany) at 50°C with the fan running at full speed for 18 h. Following drying, the stems and leaves were separated from the roots. The dried plant leaves were ground using a Tefal 8100.31 coffee and spice grinder (France). The ground samples were placed in beakers, sealed with paraffin, and kept in the refrigerator at +4°C for future use.

Figure 1. Images of the leaves used in the study.

Bioactive properties

Extraction

Bioactive extracts were prepared by soaking herb powder samples in absolute ethanol at a ratio of 1:10 (w:v) for 5 h at room temperature, following the method outlined by Kilicli et al. (2023). After extraction, the mixture was centrifuged at 5000 rpm for 5 min at room temperature. The resulting supernatant was then concentrated using a rotary evaporator and

subsequently lyophilized to yield the dry extract.

Total phenolic content (TPC)

TPC of the samples was determined using the method described by Yasar et al. (2022). Specifically, 0.5 mL of extract was combined with 2.5 mL of 0.2 N Folin–Ciocalteu reagent and mixed thoroughly. After 3 min, 2 mL of 7.5% Na_2CO_3 was added to the mixture. Subsequently, the solution was then incubated in the dark at room temperature for half an hour. The absorbance was measured at 760 nm using a UV/VIS spectrophotometer (Shimadzu UV-1800, Japan), and the results were reported as milligrams of gallic acid equivalent (GAE) per gram.

Antioxidant activity assays

ABTS

The ABTS value of leaves was measured using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid). To perform the analysis, x mL of the sample solution was combined with (4 − x) mL of ethanol, while 4 mL of ethanol alone served as the reagent blank. Subsequently, 1 mL of a 1:10 diluted ABTS radical cation solution was added at 15-sec intervals and mixed thoroughly. The ABTS solution was prepared by mixing a 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the reaction to proceed in the dark at room temperature for 12–16 h. After incubation for 6 min, the absorbance of the mixture was measured using a spectrophotometer (Shimadzu UV-1800, Nippon, Japan) at 734 nm, with ethanol used as the reference. The antioxidant capacity of the tested samples was calculated based on a Trolox calibration curve and recorded as milligrams of Trolox equivalents per g of dry extract (mg TE g^{-1} dry extract) (Erol et al., 2023).

Iron (III) ion reducing antioxidant power (FRAP) assay

To achieve this, 0.1 mL of the herb sample was mixed with 3 mL of freshly prepared FRAP reagent, which consisted of 300 mmol L^{-1} acetate buffer (pH 3.6), 20 mmol L^{-1} ferric chloride, 10 mmol L^{-1} TPTZ, and 25 mmol L^{-1} HCl. The mixture was homogenized using a vortex mixer and incubated at 25°C for 10 min. After incubation, the absorbance of the solution was measured spectrophotometrically at 593 nm using a Shimadzu UV-1800 spectrophotometer (Kutlu, 2024).

Determination of enzyme inhibition activity

To assess the inhibitory activities of the samples against AChE/BChE, an *in vitro* method was utilized following the procedure outlined by Bozkurt et al. (2021). Galantamine was used as positive control, while buffer blanks acted as reference points. The inhibitory effects of the leaf extracts on alpha-glycosidase and alpha-amylase were measured using the approach described by Kutlu (2024), with acarbose serving as the positive control and phosphate buffer as the negative control instead of the sample.

Determination of anticancer activity

CaCo-2 (ATCC, HTB-37) and HEK-293 cell lines (ATCC, CRL-1573) which is a healthy cell line, were sourced from the American Type Culture Collection (Bozkurt et al., 2021). The *in vitro* cytotoxicity assay was carried out with slight adjustments using the MTT method, as described by Kutlu (2024). To assess cytotoxicity, 15 x 10^3 cells per well were seeded in 96-well plates and cultured for 24 h. After removing the culture medium, different concentrations of cell-free filtrate and lyophilized filtrate were added, with noninoculated DMEM F-12 medium serving as a negative control. For CaCo-2 cells, incubation continued for an additional 8 and 24 h. Following this, 100 µL mL^{-1} of MTT solution was added to each well, and after 2 h of incubation at 37°C, 100 µL of dimethyl sulfoxide (DMSO) was used to dissolve the blue crystals. Absorbance was measured at 570 nm using a microplate reader (Bio-Tek, ELX808IU, USA).

Statistical analysis

The study was conducted in triplicate, and quantitative data were presented as mean ± standard deviation. The Shapiro-Wilk W test was employed to evaluate the normality of the data

distribution, where a p-value of ≥ 0.05 was considered indicative of a normal distribution. The results were analyzed using analysis of variance (ANOVA), and the using Tukey's honestly significant difference (HSD) test was employed to assess the significance of differences between the parameters of the tested leaves, with analysis performed using JMP (SAS Institute, Inc., Cary, USA) software.

Results and Discussion

Bioactive properties

Phenolic compounds are crucial for the antioxidant activity of plants, and quantifying their content is vital for assessing their antioxidant potential. These compounds provide multiple benefits, including antioxidant, antimicrobial, and anticancer properties, contributing to the prevention of diabetes, cancer, cardiovascular diseases, and oxidative stress-related conditions (Ghevariya et al., 2023; Hajra & Paul, 2018). In this context, the TPC levels (mg GAE g^{-1} in DW) of various leaf samples were ranked in descending order as follows: NL (241.86) > 165.68 (WBL)> 72.09 (FL) as shown in Table 1.

Table 1. TPC, FRAP and ABTS radical cation scavenging abilities of fenugreek (FL), white mulberry (WBL), and nettle leaves (NL).

Type of leaf	TPC (mg GAE g^{-1} DW)	Antioxidant activities	
		FRAP (µmol Fe^{2+} g^{-1} extract)	ABTS (mg TE g^{-1} extract)
FL	72.09±6.81^{c}	125.66±4.04^{b}	8.51±1.15^{b}
WBL	165.68±22.81^{b}	238.87±2.16^{a}	17.87±2.39^{a}
NL	241.86±20.98^{a}	240.48±4.66^{a}	19.26±2.86^{a}

Statistical differences within the same column are indicated by lower case letters ($^{a-c}$). The differences in TPC and FRAP results were highly significant at the p<0.001 level, while the ABTS activity results demonstrated significance at the p<0.01 level.

A statistically significant difference was found among all samples (p<0.001). In this context, Salam et al. (2023) reported a TPC of 15.27 mg GAE g^{-1} DW in crude extracts from air-dried fenugreek leaves. Similarly, Ghevariya et al. (2023) found the TPC in dried fenugreek leaves to be 53.94 mg GAE/g DW. Moreover, Hafeez et al. (2023) further highlighted that the TPC of different extract fractions (methanol, ethanol, n-hexane, and aqueous) from *T. foenum-graecum* leaves ranged from 5.74 to 6.23 mg GAE 100 g^{-1}. Meanwhile, Wang et al. (2021a) demonstrated that mulberry leaves possess a wide range of phenolic compounds, with their TPC varying between 2.61 and 51.81 mg g^{-1} DW, influenced by cultivar and geographic origin. As well, Iqbal et al. (2012) reported a TPC of 16.21 mg GAE g^{-1} DW in dried mulberry leaves of *M. alba*. Additionally, Shonte et al. (2020) observed TPC values of 118.4 mg GAE g^{-1} DW in fresh stinging NL, 121.5 mg GAE g^{-1} DW in freeze-dried leaves, and 128.7 mg GAE g^{-1} DW in oven-dried leaves.

Antioxidants neutralize free radicals in the body, preventing oxidative damage from pollution, metabolism, and external factors, which can otherwise lead to premature aging, cardiovascular disease, and degenerative conditions such as cancer, Alzheimer's, and cataracts; thus, natural antioxidants in foods like fruits, vegetables, and plant-based diets are essential for disease prevention (Ghevariya et al., 2023). The antioxidant activities of plant samples are known to be influenced by various factors, such as the extraction method, solvent used, and the assay system employed. Consequently, it is important to evaluate antioxidant activity using different assays to account for the various types of antioxidant effects (Wang et al., 2021b). The ABTS radical cation ($ABTS^{\cdot+}$) is produced via enzyme or chemical processes. Due to the ability of $ABTS^{\cdot+}$ to dissolve in both aqueous and organic solvents, it reflects the hydrophilic and lipophilic properties of sample

compounds. In contrast, the ferric reducing antioxidant power (FRAP) assay measures the reduction of the Fe^{3+} complex of tripyridyltriazine $(TPTZ)^{3+}$ to the deeply blue Fe^{2+} complex $(TPTZ)^{2+}$ by antioxidants in an acidic environment (Salam et al., 2023). The use of both FRAP and ABTS tests allows for a more comprehensive assessment of the reaction kinetics and responses of different radicals and phenolic compounds (Iqbal et al., 2012). The ABTS assay was used to evaluate the free radical scavenging activities of the leaves, and the FRAP assay was employed to measure their reducing power. The FRAP and ABTS assay results to assess the antioxidant potential of the leaf extracts for different leaf extracts are presented in Table 1. Accordingly, the ABTS antioxidant activities of the other ethanolic leaf extracts, ranked from highest to lowest, are NL (19.26 mg TE g^{-1} extract) > WBL (17.87 mg TE g^{-1} extract) > FL (8.51 mg TE g^{-1} extract). There is no statistical difference between NL and WBL ($p \geq 0.05$), while there is a statistical difference between FL and the other two ($p < 0.05$). Furthermore, Iqbal et al. (2012) reported that mulberry leaves exhibited ABTS radical cation scavenging activity, with values of 6.12mM Trolox equivalent for *M. alba.* Moreover, ABTS activity in mulberry leaves was 70.30 for the methanolic extract and 82.53 mg TE g^{-1} for the water extract, while FRAP activity was 70.16 for the methanolic extract and 59.54 µM TE g^{-1} for the water extract (Uysal et al., 2016). In addition, Eruygur and Dural (2019) stated that the antioxidant potential of mulberry leaves is primarily attributed to their polyphenolic compounds, including flavonoids and anthocyanins.

Table 1 displays the chelating activity of various leaf extracts on ferrous ions. In this assay, all leaf extracts inhibited the formation of the ferrous-ferrozine complex, indicating their chelating ability to capture ferrous ions before the complex could form. The FRAP activity peaked at 240.48 µmol Fe^{2+} g^{-1} extract in the NL samples, while it was lowest at (125.66 µmol Fe^{2+} g^{-1} extract) in the FL samples. No statistical difference was found between FBL and NL ($p > 0.05$). Wang et al. (2021a) observed that FRAP values ranged from 35.13 µmol Fe^{2+} g^{-1} DW (G1) to 227.8 µmol $Fe^{2+}g^{-1}$ DW, and ABTS values varied between 19.81 µmol TEAC g^{-1} DW and 120.42 µmol TEAC g^{-1} DW. They also found a significant positive correlation between the free phenolic content and antioxidant activity. Wang et al. (2021b) observed that, regardless of the solvent used, the free phenol content showed the strongest relative correlation with ABTS compared to FRAP in mulberry leaves. The FRAP and ABTS values were reported as 258.864 mmol Fe^{2+} g^{-1} and 0.097 mmol g^{-1}, respectively (Zhang et al., 2018). The FRAP activity of NL was reported to range between 9.5 and 75.5 µmol Fe^{2+} g^{-1} DW, depending on the extraction solvent and duration (Vajić et al., 2022). Moreover, the variations in test results may be due to different kinetics of reactions between radicals and phenolic compounds or differing responses of phenolics to various radicals. Therefore, employing a range of antioxidant tests together is important for accurately evaluating the antioxidant capacity of a sample (Iqbal et al., 2012). In this study, a positive correlation between TPC and antioxidant activity values has been identified. Likewise, Salam et al. (2023) identified a linear relationship between TPC and ABTS scavenging activities, as well as FRAP activity. In summary, the high levels of phenolics, combined with substantial FRAP and ABTS radical cation scavenging potential, indicate that NL is superior to other species in terms of its disease-preventive potential.

Enzyme inhibitory properties

Antidiabetic potential of leaf extracts

Phytochemicals have garnered significant interest for diabetes treatment due to their potential benefits, leading many researchers to focus on extracting hypoglycemic agents from medicinal plants. Among these, plant polyphenols and flavonoids are recognized natural antidiabetic agents that inhibit carbohydrate-hydrolyzing enzymes by binding with proteins, thereby helping to reduce postprandial hyperglycemia in diabetes (Ganeshpurkar et al., 2013). In this context, Table 2 presents the antidiabetic potential of leaf extract, revealing that the inhibition of alpha-amylase

ranges from 8.85% to 90.39% depending on extract concentration in the range of 62.5-500 µg mL^{-1}.

Table 2. Antidiabetic activities of fenugreek (FL), white mulberry (WML), and nettle leaves (NL).

Enzyme type	Concentration (µg mL^{-1})	Inhibition percentages					
		62.5 (µg mL^{-1})	125 (µg mL^{-1})	250 (µg mL^{-1})	400 (µg mL^{-1})	500 (µg mL^{-1})	IC_{50} (µg mL^{-1})
Alpha-amylase	FL	8.85±0.56^{De}	20.34±1.00^{Dd}	44.17±1.42^{Dc}	58.17±2.33^{Cb}	62.28±2.65^{Ca}	357.85
	WBL	19.24±0.47^{Ae}	36.29±1.60^{Ad}	64.24±1.46^{Ac}	83.31±3.82^{Ab}	90.39±2.21^{Aa}	213.99
	NL	14.24±0.43^{Be}	32.88±0.78^{Bd}	56.51±1.34^{Bc}	75.14±1.62^{Bb}	86.87±2.45^{Aa}	248.01
	Acarbose	12.53±0.33^{Ce}	28.77±0.21^{Cd}	53.81±0.29^{Cc}	70.89±2.01^{Bb}	79.34±0.53^{Ba}	273.67
Alpha-glucosidase	FL	13.44±0.95^{Be}	25.57±0.72^{Dd}	39.69±0.69^{Dc}	50.91±2.69^{Cb}	58.31±5.11^{Ca}	393.63
	WBL	17.55±1.42^{Ae}	39.36±2.07^{Ad}	66.37±2.62^{Ac}	81.24±3.17^{Ab}	86.73±2.15^{Aa}	213.58
	NL	17.47±1.71^{Ae}	35.20±0.54^{Bd}	59.41±1.24^{Bc}	75.96±3.03^{Ab}	82.06±1.88^{Aa}	239.83
	Acarbose	14.15±1.13^{Be}	32.27±1.76^{Cd}	51.44±2.84^{Cc}	70.09±2.66^{Bb}	74.97±2.27^{Ba}	277.78

Differences among leaf extract concentrations in enzyme inhibition percentages are indicated by different capital letters within the same column, with a significance level of $p<0.01$. Differences in leaf samples within the same row are indicated by different lowercase letters ($p<0.05$).

The inhibitory potential of WBL (IC_{50} = 213.99 µg mL^{-1}) and NL (IC_{50} = 248.01 µg mL^{-1}) was lower than that of acarbose (IC_{50} = 273.67 µg mL^{-1}) but higher than FL (IC_{50} = 357.85 µg mL^{-1}). Our results show that leaf samples WBL and NL exhibited significantly higher *in vitro* alpha-amylase inhibitory activity compared to sample FL, which showed lower activity (see Table 2). Acarbose, a complex oligosaccharide, delays carbohydrate digestion by inhibiting amylase, but synthetic inhibitors can lead to side effects such as abdominal pain, diarrhea, and soft stools (Narkhede, 2012). In this context, Hafeez et al. (2023) found that the inhibition of alpha-amylase by *T. foenum-graecum* leaf extract ranged from 9.43% to 24.95% depending on different extract fractions. Moreover, at a concentration of 250 µg mL^{-1}, the ethyl acetate extract demonstrated significant alpha-amylase inhibition at 64.55% and alpha-glucosidase inhibition at 52.56% (Ganeshpurkar et al., 2013). Alpha-amylase inhibitors act as anti-nutrients by impeding the digestion and absorption of carbohydrates, which can be beneficial in managing obesity and diabetes (Narkhede, 2012). Although the exact mechanisms through which plant protein inhibitors affect alpha-amylase are not fully understood, it is suggested that plant proteins, particularly flavanols, may induce structural changes in the enzyme. Additionally, plant phenolic compounds have been shown to influence carbohydrate breakdown by inhibiting amylase activity. The observed activity in the selected leaf samples may be attributed to the presence of tannins in their ethanol extracts (Narkhede, 2012). These results suggest that the plant may exhibit hypoglycemic activity, likely through the inhibition of alpha-amylase. This effect could be partly attributed to reduced glucose absorption into the bloodstream due to the alpha-amylase inhibitory activity of the plant extract. Additionally, improved glucose tolerance may be linked to other mechanisms such as stimulation of glycogenesis in the liver, increased tissue glucose utilization, and decreased gluconeogenesis. Phytoconstituents such as flavonoids, polyphenols, tannins, alkaloids, glycosides, carbohydrates, and proteins have been shown to possess antidiabetic activity (Bisht et al., 2021). A previous study indicated that oxidative stress may contribute to increased insulin resistance, suggesting that antioxidants could be useful in treating diabetes (Hajra & Paul, 2018). However, in our study, no correlation was found between alpha-amylase and alpha-glucosidase enzyme inhibition and IC_{50} values.

Alpha-glucosidase is an enzyme that breaks down complex carbohydrates into glucose by hydrolyzing the terminal 1,4-glycosidic bonds. This

enzyme is located in the brush-border epithelium of the human intestine. Conventional inhibitors of alpha-glucosidase are less effective at inhibiting this enzyme compared to their impact on alpha-amylase, which is primarily responsible for gastrointestinal discomfort in individuals with diabetes (Wadhawan, et al., 2018). To reduce this side effect, it's essential to investigate alternative herbs and plants as safer sources of alpha-glucosidase inhibitors. In this context, the alpha-glucosidase inhibition activity of WBL (19.24-90.39%) and NL (14.24-86.87%) was observed to be higher than that of the positive control acarbose (12.53-79.39%) as shown in Table 2. Among the samples mentioned, WBL and NL were identified as having a significant inhibitory effect on alpha-glucosidase at the concentration ranges of 62.5-500 µg mL^{-1}. In contrast, FL (13.44-58.31%) did not exhibit significant alpha-glucosidase inhibitory activity. Differences in enzyme inhibitory activity may arise from the varying abilities of solvents to extract specific compounds based on their chemical nature, physicochemical properties such as polarity, and the presence or absence of interfering substances (Sharma et al., 2023). Accordingly, Kim et al. (2011) found that mulberry leaf extract exhibited strong *in vitro* inhibition of intestinal alpha-glucosidase, while its effect on intestinal alpha-amylase was significantly weaker in comparison to acarbose. A recent study has shown that 1-deoxynojirimycin (DNJ) and its derivatives, which are primary constituents of mulberry leaves, effectively inhibit intestinal alpha-glucosidases, thereby slowing down the digestion of carbohydrates (Oku et al., 2016). Additionally, Eruygur and Dural (2019) suggested that the enzyme inhibition activity observed could be linked to the polyhydroxylated alkaloids and specific phenolic compounds found in mulberry extracts from Türkiye. Similar to acarbose, the inhibitory potential of mulberry leaf extracts may delay the metabolism of saccharides, reduce glucose absorption, and thus help manage postprandial blood sugar levels. Moreover, Han et al. (2020) identified alkaloids and flavonoids in mulberry leaf powder as the key agents in combating type 2 diabetes, with alkaloids demonstrating a pronounced inhibition of alpha-glucosidase activity, and flavonoids showing secondary effectiveness. In addition, Habeeb et al. (2012) found that the 60% mulberry alcohol extract had the greatest alpha-glucosidase inhibition at 80.92%, in contrast to the 100% alcohol extract, which had the lowest inhibition of 30%. At a concentration of 1 mg mL^{-1}, the 60% mulberry alcohol extract provided 69.69% inhibition of salivary amylase and 78.77% inhibition of pancreatic amylase, whereas the 100% alcohol extract showed a minimal inhibition of 16.58%. Furthermore, Adisakwattana et al. (2012) found that mulberry leaf extract exhibited the strongest inhibitory effect on intestinal alpha-glucosidase but showed no inhibition of pancreatic alpha-amylase. In this experiment, the leaf extracts demonstrated notable inhibition of alpha-amylase and alpha-glucosidase in a dose-dependent manner.

Anti-Alzheimer potential of leaf extracts

Alzheimer's disease, the most common neurological disorder, currently has limited treatment options. Acetylcholinesterase activity is associated with Alzheimer's disease and can be inhibited by certain medicinal plants. Plant-derived treatments offer neuroprotective benefits that could help manage neurodegenerative conditions (Hafeez et al., 2023). The enzyme inhibitory activities of the leaves against AChE and butyrylcholinesterase (BChE) are summarized in Table 3.

Table 3. Anti-Alzheimer enzyme inhibition activities of various leaves.

Type of wastes	Anti-Alzheimer activity	
	AcHE (%)	BcHE (%)
FL	39.93±1.35^{c}	33.87±3.60^{b}
WBL	23.14±1.74^{d}	17.94±1.40^{c}
NL	53.35±1.93^{b}	38.40±2.62^{b}
Galanthamine	90.28±3.92^{a}	87.12±5.24^{a}

FL: Fenugreek leaf; WBL: White mulberry leaf: WBL; NF: Nettle leaf.
Statistical differences within the same column are indicated by lower case letters ($^{a-d}$) ($p<0.001$).

The AChE inhibition rates for all tested samples of leaves ranged from 23.14% (WBL) to 53.35% (NL). Conversely, the findings reveal that ascorbose achieved a maximum inhibition rate of 90.28% on acetylcholinesterase, whereas none of the leaf extracts demonstrated any inhibition against AChE.

Table 3 illustrates the impact of leaf extracts on inhibiting BChE activity. Among the extracts, NL ethanolic extracts (38.40%) demonstrated the highest BChE inhibitory activity compared to the FL (33.87%) and WBL (17.94%) at 2 mg mL^{-1}. None of the leaf extracts showed inhibition against BChE, as their BChE inhibition percentages were lower than the inhibition percentage of the galanthamine. at 2 mg mL^{-1}. In a related study, Uysal et al. (2016) reported that in mulberry leaves, the AChE and BChE inhibitory activities were higher in ethanol extracts compared to water extracts. BChE activity was observed around 30%, while AChE activity was found to be in the range of 20%.

Cytotoxic properties

Table 4 shows the viability of HEK-293 cells treated with FL, WBL, and NL ethanol extracts, with data presented as percentages normalized to the untreated control. After 24 h of incubation, all leaf ethanol extracts decreased the viability of HEK-293 cells from 100% to 59.33% for FL, from 100% to 61.67% for WBL, and from 100% to 55.33% across the concentration range of 1–1000 µg mL^{-1} (see Table 4). The ethanol extracts from FL, WBL, and NL yielded IC_{50} values of 1159.98 µg mL^{-1}, 1235.67 µg mL^{-1}, and 972.22 µg mL^{-1} on HEK-293 cells, respectively. Notably, the NL extract, with the lowest IC_{50} value, exhibits the strongest inhibitory effect on the HEK-293 cells. This implies that the NL extract may offer a higher level of biological activity compared to the other two extracts.

Table 4 presents the viability percentages and IC_{50} values of leaf extracts for the CaCo-2 cells. The data indicates that the FL extracts exhibited a range of cell viability from 100% to 51.00% across the concentration range of 1–1000 µg mL^{-1}.

Table 4. The percentage of cell viability and IC_{50} values for FL, WBL, and NL.

Name of cells	Sample name	Control	1 (µg mL^{-1})	50 (µg mL^{-1})	100 (µg mL^{-1})	250 (µg mL^{-1})	500 (µg mL^{-1})	1000 (µg mL^{-1})	IC_{50} (µg mL^{-1})
HEK-293	FL	100.00±0.00^{Aa}	100.00±0.00^{Aa}	95.67±1.53^{Aab}	89.28±1.53^{Ab}	82.33±5.86^{Ac}	73.33±1.53^{Ad}	59.33±1.53^{ABe}	1159.98
	WBL	100.00±0.00^{Aa}	100.00±0.00^{Aa}	94.00±1.73^{Ab}	85.26±1.53^{Ab}	77.33±3.51^{ABc}	75.33±0.58^{Ac}	61.67±2.08^{Ad}	1235.67
	NL	100.00±0.00^{Aa}	100.00±0.00^{Aa}	85.33±3.06^{Bb}	86.45±0.58^{Bbc}	73.00±3.61^{Bc}	62.33±2.52^{Bd}	55.33±3.06^{Bd}	972.22
Caco-2	FL	100.00±0.00^{Aa}	100.00±0.00^{Ba}	89.00±2.00^{Ab}	83.67±2.31^{Ac}	72.00±2.65^{Ad}	62.33±1.53^{Ae}	51.00±1.00^{Af}	897.41
	WBL	100.00±0.00^{Aa}	100.00±0.00^{Ba}	86.67±2.08^{Ab}	79.33±1.15^{Bc}	68.00±3.61^{Ad}	53.67±2.08^{Be}	44.67±3.21^{Bf}	754.11
	NL	100.00±0.00^{Aa}	102.00±1.00^{Aa}	81.67±0.58^{Bb}	75.00±2.00^{Cc}	61.67±2.08^{Bd}	44.33±3.06^{Ce}	40.33±1.15^{Cf}	648.08

Fenugreek leaf: FL; White mulberry leaf: WBL; Nettle leaf: NL.
Differences among leaf extract concentrations for the cell viability findings are represented by different capital letters within the same column, with a significance level of $p<0.01$. Differences in leaf samples within the same row are indicated by different lowercase letters ($p<0.05$).

In comparison, WBL extracts showed cell viability values ranging from 100.00% to 44.67% within the same concentration range, while NL extracts resulted in cell viability ranging from 40.33% to 100%. In both cells, an increase in extract concentration resulted in a decrease in cell viability percentage. The IC_{50} values, which reflect the concentration required to inhibit 50% of cell

viability, were calculated as follows: 897.41 µg mL^{-1} for FL, 754.11 µg mL^{-1} for WBL, and 648.80 µg mL^{-1} for NL. These findings suggest that among the leaf extracts tested, NL exhibits the lowest IC_{50} value, indicating the highest potency in reducing cell viability. Conversely, FL and WBL extracts demonstrated higher IC_{50} values, implying relatively lower efficacy compared to NL. The observed differences in IC_{50} values highlight the varying degrees of biological activity among the extracts. NL's lower IC_{50} value suggests it may have a more significant impact on cell viability, potentially making it a more effective candidate for further investigation. In this regard, Salam et al. (2023) found that the highest cytotoxic activity (89.03%) was observed in the crude extract of air-dried fenugreek leaves when tested on the RAW 264.7 cells at a concentration of 100 µg mL^{-1}. Moreover, Wadhawan et al. (2018) observed that the fenugreek microgreen extract was well-tolerated by HepG2 (human liver cancer cell line) cells at a concentration of 20 mg mL^{-1}. However, it was found to be mildly cytotoxic in L6 (rat myoblast) cells at concentrations above 15 mg mL^{-1}. In comparison, fenugreek seed extract has been reported to be toxic at higher concentrations, with an IC_{50} of 1 mg mL^{-1} in HepG2 cells and 2 mg mL^{-1} in L6 cells (Kadan et al., 2013; Khalil et al., 2015). Khoja et al. (2022) found that fenugreek methanolic extracts had dose and time-dependent effects on the viability of MCF-7 cells. Treatment with these extracts resulted in heightened relative mitochondrial DNA damage in the MCF-7 cancer cells, along with reduced metastasis and cell proliferation. Likewise, Yamamoto et al. (2017) investigated DNJ (1-deoxynojirimycin)'s impact from mulberry leaves on male mice with azoxymethane-induced colorectal cancer, revealing that DNJ suppressed tumor growth by promoting apoptosis, potentially through the Bcl-2/Bax signaling pathway. Additionally, Deepa et al. (2013) reported that leaf extracts from *Morus alba* can induce cytotoxic effects in human colon cancer (HCT-15) cells (IC_{50} = 13.8 µg mL^{-1}) and MCF-7 cells (IC_{50} = 9.2 µg mL^{-1}), resulting in significant DNA fragmentation, caspase-3 activation, and morphological changes in the cells, which are characteristics of apoptosis. Moreover, Deepa et al. (2012) found that the anti-proliferative lectin (MLL) derived from *M. alba* leaves caused notable morphological alterations and DNA fragmentation linked to apoptosis in MCF-7 cells. Moreover, Fattahi et al. (2014) noted that BT-474 cell viability was stable at extract concentrations up to 1.5 mg mL^{-1} but decreased to below 50% of the control at 3 mg mL^{-1}, whereas Hela cells showed no significant changes even with 3 mg mL^{-1} of extract over 3 days; furthermore, no significant differences in cell survival rates were observed between consecutive days for both Hela and BT-474 cells ($P>0.05$). Moreover, NL extracts have shown cytotoxic effects against Hep2c, RD, and L2OB cells, with activity varying depending on the extraction technique (16.73-.29.77 µg mL^{-1}) (Zeković et al., 2017). These results indicate that the tested leaf extracts have low toxicity levels on both HEK-293 and CaCo-2 cells, with minimal toxic effects on the cells; furthermore, this effect is lower in the HEK-293 cell.

Conclusions

M. alba L., *T. foenum-graecum* L., and *U. dioica* L. are traditionally recognized for their medicinal and nutritional benefits, being incorporated into diets in the form of herbal teas and other supplements for their health-promoting qualities. This study aimed to assess the total phenolic content (TPC) and antioxidant properties, including FRAP and ABTS activities, of ethanolic extracts from fenugreek (FL), white mulberry (WBL), and nettle leaves (NL). Additionally, it investigated the extracts' inhibitory effects on key enzymes—alpha-amylase, alpha-glucosidase, acetylcholinesterase, and butyrylcholinesterase—and evaluated their cytotoxicity on the HEK-293 and Caco-2 cells. The results indicated that NL exhibited superior disease-preventive potential due to its high phenolic content and strong FRAP and ABTS radical cation scavenging abilities. A positive correlation was found between total phenolic content and antioxidant activity values. Moreover, WBL and NL demonstrated significant

inhibitory properties against both alpha-amylase and alpha-glucosidase. In terms of cytotoxicity, NL showed the highest toxicity and FL the lowest in HEK-293 cells, while in the CaCo-2 cellse, NL again exhibited the highest toxicity and FL showed the lowest. These findings highlight the potential of these leaf extracts in developing functional foods aimed at enhancing the inhibition of intestinal alpha-glucosidase and alpha-amylase. Future research should focus on exploring the *in vivo* effects of these activities to further validate their therapeutic potential. While this study provides valuable insights into the phenolic content, antioxidant activities, and enzyme inhibitory properties of the extracts, it is limited by its *in vitro* nature and the restricted range of cell lines tested. Further *in vivo* studies and a broader exploration of geographical and seasonal variations are needed to confirm these findings.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Author Contributions

Kubra Feyza EROL: Methodology, validation, investigation. **Gozde KUTLU:** Investigation, Writing – original draft, review & editing.

Data Availability Statement

Data will be made available on request upon reasonable request from the corresponding author.

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