

Comparative Evaluation of Antioxidant and Enzyme Inhibitory Activities of Pomegranate Vinegars and Extracts

Güleyfe Beyza Yıldırım ¹, Çağlayan Ünsal Gürer ², Ismail Yener ³, Mehmet Akdeniz ³, Serkan Yigitkan ⁴, Mustafa Abdullah Yılmaz ³, Suzan Tireki ⁵, Abdulsalam Ertas ³ and Aslı Barla Demirköz ^{6,7*}

¹Istanbul Okan University, Faculty of Health Sciences, Department of Nutrition and Dietetics, 34959 Istanbul, Türkiye

²Istanbul University, Faculty of Pharmacy, Department of Pharmacognosy, 34116 Istanbul, Türkiye

³Dicle University, Faculty of Pharmacy, Department of Analytical Chemistry, 21280 Diyarbakir, Türkiye

⁴Dicle University, Faculty of Pharmacy, Department of Pharmacognosy, 21280 Diyarbakir, Türkiye

⁵Ozyegin University, Faculty of Applied Sciences, Gastronomy and Culinary Arts Department, 34794 Istanbul, Türkiye

⁶Istanbul Okan University, Faculty of Pharmacy, Department of Analytical Chemistry, 34959 Tuzla, Istanbul, Türkiye

⁷AROMSA Research and Development Center, Aromsa Flavours and Food Additives San. ve Tic. A.S., 41480 Kocaeli, Türkiye

(Received October 06, 2025; Revised October 24, 2025; Accepted October 27, 2025)

Abstract: In this study, antioxidant potential and enzyme inhibitory activities of pomegranate vinegars and extracts were investigated. Antioxidant activity was evaluated using ABTS, DPPH, and CUPRAC assays. Total phenol and flavonoid contents were also determined spectrophotometrically. The enzyme inhibition analysis comprised the assessment of anticholinesterase, anti-urease, and anti-aging (tyrosinase, elastase, and collagenase) targets. Pomegranate vinegars showed significantly higher total phenolic and total flavonoid contents with respect to crude extracts. The antioxidant activity of vinegar samples was higher, and one of the investigated vinegars (vinegar 2) exerted the highest radical-scavenging ability (DPPH IC₅₀: 2.21±0.01 µg/mL). Enzyme inhibition assays demonstrated that vinegars showed a broad-spectrum activity against several targets. The maximum anticholinesterase activity (AChE: 96.53±1.47%; BChE: 94.35±0.74%) was observed by one of the vinegars (vinegar 1), exceeding that of galantamine. Another vinegar sample (vinegar 3) had remarkable elastase (96.57±1.80%) and collagenase inhibition. All vinegars exhibited potent inhibitory activity against α-glucosidase (up to 110.97±3.01%), significantly higher than acarbose. On the other hand, ethanolic extracts displayed weak or no inhibitory activities. Fermentation markedly improved the phenolic profile and multifunctional bioactivities of pomegranate products, providing vinegars with potent antioxidant, neuroprotective, anti-aging, and anti-urease activities. These results reveal pomegranate vinegar as a new functional food candidate with potent nutraceutical activities.

Keywords: *Punica granatum*; pomegranate vinegar; antioxidant capacity; enzyme inhibitory activity. © 2025 ACG Publications. All rights reserved.

*Corresponding author: E-Mail: aslibarla@gmail.com

1. Introduction

Functional foods prepared from fruits rich in bioactive compounds have recently gained popularity. In this regard, pomegranate (*Punica granatum* L.) is noteworthy due to its rich phenolic contents, antioxidant capacity, and wide range of health-beneficial effects [1, 2]. Although pomegranate is a fresh fruit or juice traditionally used, it has also been developed into value-added products (wine, vinegar, and dietary supplements), offering potential for innovation in the functional food market [3].

Using a sequential alcoholic and acetic fermentation, pomegranate vinegar keeps a high proportion of fruit polyphenolic fraction of interest, such as ellagic acids and their derivatives, and anthocyanins, which are recognized for their high antioxidant and antimicrobial potential [4,5]. Pomegranate vinegar has been reported to have the same or even higher antioxidant activity than apple and wine vinegars in comparative analysis [6, 7].

Furthermore, bioactive compounds in arils and by-products (skins and seeds) enhance their nutraceutical potential. Pomegranate peel extracts, rich in ellagitannins and flavonoids, are particularly abundant, contributing to their radical-scavenging and anti-inflammatory properties [4, 8]. Moreover, other investigations have reported that pomegranate phenolics can also be cardioprotective, antidiabetic, and anti-carcinogenic, endorsing the functionality of pomegranate vinegar as a dietary ingredient [2, 3].

Beyond its culinary value, vinegar has been used for many years to treat a variety of diseases, with antihypertensive, anti-diabetic, anti-obesity, lipid-lowering, and antimicrobial properties. [9, 10]. The consumption of vinegar has been shown to have therapeutic effects, including reducing postprandial blood glucose levels, modifying lipid accumulation, lowering oxidative stress, and improving inflammatory pathways, all of which are key factors in preventing chronic metabolic diseases [11, 12]. These effects are attributed to the synergistic interactions between acetic acid and phenolic compounds in regulating carbohydrate metabolism, lipid oxidation, and vascular function.

Pomegranate vinegar has shown potential applications in the treatment of obesity and diabetes through activation of AMP-activated protein kinase, inhibition of lipogenesis, and increased fatty acid β -oxidation [13, 14]. Supplement studies show that these extracts reduce circulating and hepatic triglycerides, improve insulin sensitivity, and possess anti-aging and anti-inflammatory activities, due to their high levels of ellagitannins and flavonoids. Nevertheless, cultivar variation, agroclimatic factors, and processing conditions strongly affect the phenolic composition and bioactive properties of pomegranate products [4, 5]. Accordingly, an integrated evaluation of the phytochemical profile and the biological activities of pomegranate vinegar will support health claims and improve production processes to exploit its functional properties.

From this perspective, the current study was designed to address part of these gaps and to investigate the antioxidant activity and potential health benefits of pomegranate vinegar and its extracts. Moreover, all extracts were evaluated for in vitro bioactivity, including antioxidant, anti-aging, anti-cholinesterase, and anti-urease activities. This research fills an essential gap in the literature. This is the first report on the simultaneous multiple bioactivities of pomegranate vinegar, which might provide an edge for its future potential as a next-generation functional food and nutraceutical.

2. Materials and Methods

2.1. Chemicals

Analytical grade reagents, enzymes, solvents, and substrates were purchased from Merck, Türkiye.

2.2. Sample Preparation and Extraction

Fresh pomegranate fruits were purchased from a local market in Istanbul, Türkiye. After washing and cutting into small pieces, the fruits were dried in a laboratory oven (Nüve, Türkiye) and ground into powder (Retsch GmbH, 1 mm). Extraction was conducted according to the method of Wang et al. (2013), with modifications. Powdered samples (360 g) were extracted with either ethanol (EtOH) or ethanol: water (70:30, v/v) at room temperature for 24 h, repeated three times. Filtrates were combined, concentrated under reduced pressure, and freeze-dried at -50°C , yielding 104 g of EtOH extract

(28.88%) and 66 g of ethanol: water extract (18.33%). Dried extracts were stored at -18°C until further analysis.

2.3. Vinegar Samples

Three commercial pomegranate vinegars - Vinegar 1, Vinegar 2, and Vinegar 3 - were obtained from separate manufacturers. Vinegars 1 and 2 were produced via traditional double fermentation (alcoholic and acetic), while Vinegar 3 was an industrially pasteurized product. All these commercial pomegranate vinegars, which comply with the Turkish Food Codex and ISO standards, were purchased from a local market in Istanbul, Türkiye. For comparison, vinegar from a widely distributed brand was used as a reference. Brand names have been omitted to avoid potential legal implications.

2.4. Preparation of Stock Solutions

Stock solutions of crude extracts were prepared at a concentration of 4.000 µg/mL, and serial dilutions were made according to the method described by Akdeniz et al. [15].

2.5. Antioxidant Activities

Antioxidant capacity of the samples was determined through three complementary assays: ABTS radical cation decolorization [16], DPPH free radical scavenging [17], and CUPRAC (Cupric Ion Reducing Antioxidant Capacity) [18]. Together, these approaches offer a comprehensive evaluation by addressing distinct radical species and redox pathways. α -Tocopherol and butylated hydroxytoluene (BHT) served as reference standards to ensure assay validity. For each method, IC₅₀ values were determined from serial dilutions of the samples (100, 50, 25, 10, and 1 µg/mL), enabling reliable comparisons of antioxidant activity.

We aimed to evaluate the functional potential of pomegranate vinegars in their native, ready-to-consume form, rather than solvent-based extracts. This design ensures that the obtained antioxidant data represent the actual biological activity of vinegars as consumed, in line with previous research [7, 19]. All antioxidant assays were performed directly on the vinegar matrices to preserve native composition. Matrix-matched blanks (3.5 % acetic acid) and spike-recovery tests were used to correct and validate potential pH or colorimetric interferences.

2.6. Total Phenolic and Flavonoid Contents

Total phenolic and flavonoid concentrations were quantified according to the protocols described by Moreno et al. [20] and Slinkard and Singleton [21], respectively. Phenolic content was expressed as pyrocatechol equivalents (PEs), while flavonoid content was expressed as quercetin equivalents (QEs). Standard calibration curves were established using Equations (1) and (2) for flavonoids and phenolics, respectively.

$$\text{Absorbance} = 0.0359 + 0.0874 \times \text{quercetin } (\mu\text{g}) \quad (r^2 = 0.9960) \quad (1)$$

$$\text{Absorbance} = 0.0412 + 0.0443 \times \text{pyrocatechol } (\mu\text{g}), \quad (r^2 = 0.9928) \quad (2)$$

These calibration models ensured precise and reproducible quantification for all analysed samples.

2.7. Enzyme Inhibitory Assays

2.7.1. Anticholinesterase Activity

Inhibitory effects on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were assessed following the spectrophotometric procedure of Ellman et al. [22] using galantamine as the positive control. Inhibition percentages were determined at a final concentration of 100 µg/mL with ethanol included as the solvent control.

2.7.2. Urease Inhibitory Activity

Urease inhibition was examined according to the protocol of Hina et al. [23], employing thiourea as the standard inhibitor.

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2.7.3. Anti-Aging Activity

Anti-aging activity was determined by measuring the inhibition of tyrosinase [24], elastase [25], and collagenase [26]. Kojic acid, oleanolic acid, and epicatechin gallate were used as standard references. For all enzyme assays, the percentage inhibition values were calculated with Equation (3).

$$\text{Inhibition (\%)} = 100 - \left(\frac{OD_{\text{test well}}}{OD_{\text{control}}} \right) * 100 \quad (3)$$

2.8. Statistical Analysis

All assays were performed in triplicate, and the results are expressed as mean±standard deviation. A one-way analysis of variance (ANOVA) was performed on the data using IBM SPSS Statistics 27.0.1. Comparisons of means were made at a significance level of $p = 0.05$. When significant differences were detected, the Tukey Test was used for post hoc comparisons ($p \leq 0.05$). SR Plot [27] was used for data visualization.

3. Results and Discussion

3.1. Total Phenolic and Flavonoid Contents and Antioxidant Activities

Total phenolic contents, flavonoid contents, and the antioxidant activities of pomegranate extracts (EtOH 70%) and vinegars are given in Table 1. Among all samples, pomegranate vinegar 1 exhibited the highest total phenolic content (600.36 ± 1.72 µg PEs/mg), followed by vinegar 2 (517.23 ± 5.90 µg PEs/mg) and vinegar 3 (164.68 ± 3.60 µg PEs/mg). In contrast, EtOH extract (37.66 ± 0.70 µg PEs/mg) and 70% EtOH extract (39.68 ± 0.98 µg PEs/mg) showed the lowest phenolic levels (Figure 1. (A)). For total flavonoid content, vinegar 2 recorded the highest value (345.71 ± 2.41 µg QEs/mg), followed by vinegar 3 (55.04 ± 0.98 µg QEs/mg), whereas the 70% EtOH extract contained only minimal amounts (1.00 ± 0.02 µg QEs/mg) (Figure 1. (A))

Table 1. Total phenolic, flavonoid contents, and antioxidant activities of the samples

Samples/Extracts	Total phenolic (µg PEs/mg)	Total flavonoids (µg QEs/mg)	Antioxidant activity (µg/mL)		
			DPPH (IC ₅₀)	ABTS (IC ₅₀)	CUPRAC (A _{0.5})
Pomegranate EtOH	37.66 ± 0.70^a	-	48.50 ± 0.48^a	14.70 ± 0.12^a	38.89 ± 0.98^a
Pomegranate 70% EtOH	39.68 ± 0.98^a	1.00 ± 0.02^a	46.29 ± 0.39^b	14.96 ± 0.17^a	44.36 ± 0.99^b
Pomegranate Vinegar 1	600.36 ± 1.72^b	-	3.10 ± 0.03^c	6.90 ± 0.02^b	1.00 ± 0.02^c
Pomegranate Vinegar 2	517.23 ± 5.90^c	345.71 ± 2.41^b	2.21 ± 0.01^c	7.35 ± 0.04^b	0.68 ± 0.01^c
Pomegranate Vinegar 3	164.68 ± 3.60^d	55.04 ± 0.98^c	8.99 ± 0.10^d	3.91 ± 0.01^c	3.24 ± 0.03^d
BHT	-	-	57.72 ± 0.87^e	16.77 ± 0.47^d	7.96 ± 0.06^e
α-Tocopherol	-	-	12.75 ± 0.13^f	7.13 ± 0.08^b	13.88 ± 0.15^f

Total phenolic content expressed as pyrocatechol equivalents. ($y = 0.0412 (\mu\text{g}) + 0.0443$, $r^2 = 0.9928$)

Total flavonoid content expressed as quercetin equivalents. ($y = 0.0359 (\mu\text{g}) + 0.0874$, $r^2 = 0.9960$)

Samples with common lower-case letters are not significantly different ($p > 0.05$).

Antioxidant activities, determined by DPPH, ABTS, and CUPRAC assays, varied considerably among the samples (Table 1, Figure 1. (B)). The lowest IC₅₀ values for DPPH radical scavenging were observed in vinegar 2 (2.21 ± 0.01 µg/mL), followed by vinegar 1 (3.10 ± 0.03 µg/mL). In contrast, EtOH extract (48.50 ± 0.48 µg/mL) and 70% EtOH extracts (46.29 ± 0.39 µg/mL) exhibited the highest IC₅₀ values, indicating weaker radical scavenging activities. Similarly, for ABTS radical scavenging, vinegar 3 showed the lowest IC₅₀ value (3.91 ± 0.01 µg/mL), while vinegar 1 and 2 showed IC₅₀ values of 6.90 ± 0.02 and 7.35 ± 0.04 µg/mL, respectively. For CUPRAC reducing power, vinegar 2 exhibited the lowest A_{0.5} value (0.68 ± 0.01 µg/mL), followed by vinegar 1 (1.00 ± 0.02 µg/mL) and vinegar 3 (3.24 ± 0.03 µg/mL). The EtOH and EtOH 70% extracts recorded significantly higher values (38.89 ± 0.98 µg/mL and 44.36 ± 0.99 µg/mL, respectively), indicating a lower antioxidant capacity. This study demonstrated that pomegranate vinegars contain significantly higher total phenolic and flavonoid contents, as well as stronger antioxidant activities, compared to ethanol and 70% ethanol extracts. Among the tested samples, vinegar 1 and vinegar 2 consistently exhibited the lowest IC₅₀ values in the DPPH, ABTS, and CUPRAC assays, indicating the highest radical-scavenging capacities. These results

support previous findings that fermentation can release bound phenolics and enhance antioxidant properties by forming new bioactive metabolites [6, 7]. Although the direct evaluation of vinegars may introduce minor matrix-related effects, it provides a realistic representation of their antioxidant potential. The inclusion of matrix-matched blanks, pH-controlled validations, and spike–recovery analyses ensured the robustness and reproducibility of the results.

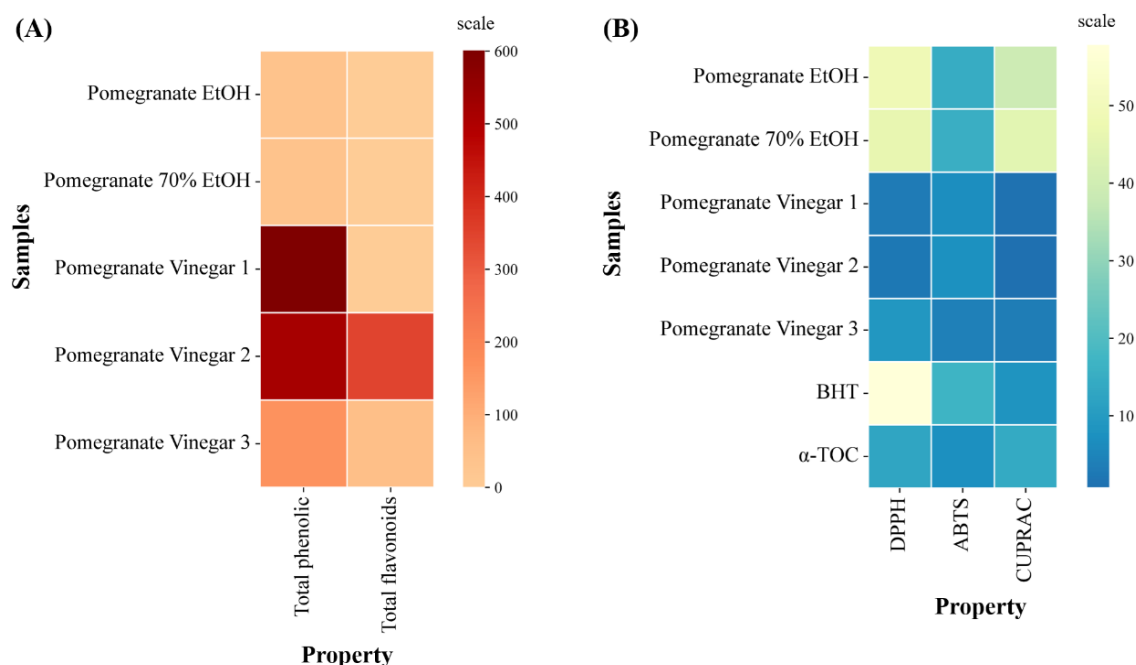


Figure 1. Matrix heatmap designation of (A) total phenolic (µg PEs/mg) and total flavonoids content (µg QEs/mg), and (B) antioxidant activity (µg/mL) in terms of DPPH (IC₅₀), ABTS (IC₅₀), and CUPRAC (A_{0.5}) for pomegranate extracts (EtOH, 70% EtOH) and vinegars.

Previous studies have reported that pomegranate-derived products rich in ellagitannins, anthocyanins, and phenolic acids show remarkable antioxidant potential [1, 3]. Pomegranate peel and juice extracts have been shown to outperform many other fruit-based products in terms of free radical scavenging and antimicrobial activities [28]. The intense activity observed in vinegar 2 may reflect both phenolic composition and fermentation parameters, factors previously identified as key determinants of antioxidant activity [3, 6].

Overall, the results highlight pomegranate vinegar as a promising functional food ingredient with potential applications in the prevention of oxidative stress-related diseases. Future research should focus on characterizing fermentation-derived phenolic compounds, assessing their bioavailability, and exploring their *in vivo* antioxidant and health-promoting effects to elucidate their functional properties [3, 28] fully.

3.2. Enzyme Inhibitory Activity Results

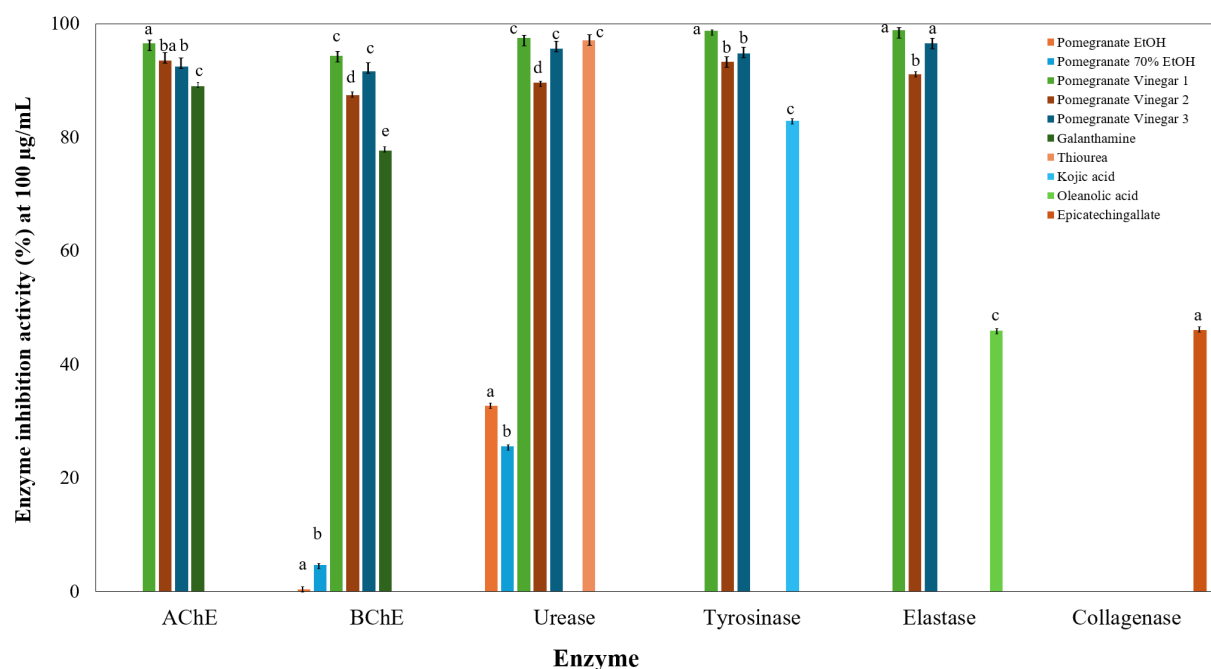
Enzyme inhibition assays revealed a marked difference between pomegranate vinegars and crude extracts (in ethanol and 70% ethanol) across all tested enzymes, as shown in Table 2 and Figure 2. While pomegranate extracts showed minimal or no inhibitory activity against most enzymes, fermented pomegranate vinegars demonstrated potent inhibition, highlighting the impact of fermentation on bioactivity.

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Table 2. Enzyme inhibition activities (%) of the samples at 100 µg/mL.

Sample	AChE Assay	BChE Assay	Urease Assay	Tyrosinase Assay	Elastase Assay	Collagenase Assay
Pomegranate EtOH	NA	0.37±0.01 ^a	32.74±1.33 ^a	NA	NA	NA
Pomegranate 70% EtOH	NA	4.68±0.06 ^b	25.55±0.77 ^b	NA	NA	NA
Pomegranate Vinegar 1	96.53±1.47 ^a	94.35±0.74 ^c	97.50±0.98 ^c	98.82±1.25 ^a	98.86±1.44 ^a	NA
Pomegranate Vinegar 2	93.57±1.05 ^{ba}	87.41±0.40 ^d	89.68±0.44 ^d	93.35±1.63 ^b	91.15±1.40 ^b	NA
Pomegranate Vinegar 3	92.47±1.41 ^b	91.61±1.16 ^c	95.62±0.88 ^c	94.83±1.05 ^b	96.57±1.80 ^a	NA
Galanthamine*	88.98±1.12 ^c	77.67±1.09 ^c	-	-	-	-
Thiourea*	-	-	97.06±1.12 ^c	-	-	-
Kojic acid*	-	-	-	82.89±1.21 ^c	-	-
Oleanolic acid*	-	-	-	-	45.96±1.32 ^c	-
Epicatechingallate*	-	-	-	-	-	46.07±0.48 ^a

*: Standard compounds; NA: Not applicable.

Samples with common lower-case letters are not significantly different ($p > 0.05$).**Figure 2.** Enzyme inhibition activities of pomegranate extracts, pomegranate vinegars, and standard compounds. Samples with common lower-case letters are not significantly different ($p > 0.05$).

3.2.1. Anticholinesterase Activity and Neuroprotective Potential

Among all samples, pomegranate vinegar 1 exhibited the highest inhibitory activity against acetylcholinesterase (AChE: 96.53±1.47%) and butyrylcholinesterase (BChE: 94.35±0.74%), surpassing even the standard inhibitor galantamine (AChE: 88.98±1.12%; BChE: 77.67±1.09%) (Figure 2). Previous studies on the anticholinesterase activity of pomegranate have generally focused on peel, leaf, or juice extracts obtained with ethanol, methanol, or aqueous-methanol (70–80%) as extraction solvents. These polar solvents efficiently extract polyphenols, hydrolysable tannins, and flavonoids, which are the primary contributors to inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [29, 30, 31]. The higher polarity of these solvents facilitates the recovery of phenolic acids and flavonoid glycosides, which are responsible for enzyme binding. In contrast, the fermented pomegranate vinegars analysed in this study represent a more complex matrix, in which fermentation induces bioconversion of ellagitannins and flavonoids into smaller derivatives such as ellagic acid, gallic acid, and catechins.

Furthermore, the fermentation process may promote partial hydrolysis of high-molecular-weight polyphenols, increasing their solubility and chemical accessibility, which could explain the more potent enzyme inhibition observed for vinegar samples compared with crude ethanolic extracts. This observation aligns with previous reports linking microbial fermentation to improved antioxidant and neuroprotective potential through phenolic transformation and enhanced bioavailability. However, no prior studies have examined the anticholinesterase activity of pomegranate vinegar. Our findings, therefore, provide the first evidence that fermentation products of pomegranate, particularly vinegar, exhibit remarkable anticholinesterase activity, suggesting potential neuroprotective applications in age-related cognitive decline and Alzheimer's disease.

3.2.2. Anti-aging Properties: Elastase, Collagenase, and Oxidative Stress

Pomegranate vinegars also demonstrated substantial inhibition of elastase and collagenase (Figure 2). Vinegar 3 showed $96.57 \pm 1.80\%$ elastase and $46.07 \pm 0.48\%$ collagenase inhibition. These enzymes are implicated in skin aging, as their overactivity leads to loss of elasticity and collagen degradation [32]. While direct studies on pomegranate vinegar are lacking, research on pomegranate extracts and fermented derivatives suggests that polyphenols such as ellagic acid and punicalagin attenuate oxidative stress, inflammation, and the formation of advanced glycation end-products (AGEs), all of which are central to the aging process [33, 34]. Our results align with these findings, suggesting that fermentation may enhance the stability of bioactive compounds, thereby strengthening their anti-aging effects.

3.2.3. Tyrosinase and Urease Inhibitory Activity

Tyrosinase inhibitory activity was highest in vinegar 1 ($98.82 \pm 1.25\%$), exceeding the standard inhibitor kojic acid ($82.89 \pm 1.21\%$) (Figure 2), indicating potential applications in the management of skin hyperpigmentation. Similarly, all vinegar samples exhibited greater than 90% inhibition of urease, an enzyme linked to gastrointestinal disorders, with vinegar 1 at $97.50 \pm 0.98\%$, comparable to thiourea ($97.06 \pm 1.12\%$). These results highlight a broad-spectrum bioactivity across multiple enzyme targets.

3.3. Impact of Fermentation on Bioactivity

The negligible activity of pomegranate EtOH and 70% EtOH extracts compared to vinegar samples underscores the critical role of fermentation. Fermentation can transform phenolic precursors into more bioactive derivatives, enhance bioavailability, and generate novel antioxidant and anti-inflammatory compounds [34]. Consequently, the superior bioactivity of pomegranate vinegars observed in this study may result from microbial biotransformation during acetic fermentation, enriching the phenolic profile beyond that of fresh extracts. Phenolic acids, such as ellagic and gallic acids, are known to inhibit acetylcholinesterase via hydrogen bonding and π - π stacking interactions with the catalytic site [35]. Similarly, flavonols such as quercetin and kaempferol may modulate α -glucosidase activity by binding to the enzyme's active pocket, thereby reducing carbohydrate hydrolysis. These mechanisms may explain the broad-spectrum enzyme inhibition observed in fermented pomegranate vinegars.

Fermentation enhances phenolic bioavailability by converting glycosylated and polymeric forms into smaller, more absorbable molecules. The acetic fermentation of pomegranate may promote the hydrolysis of ellagitannins into ellagic acid and its derivatives, thereby increasing intestinal permeability and potentially affecting systemic health. However, these transformations require further *in vivo* verification to confirm their nutritional relevance.

4. Conclusion

The present study is the first evidence that the functional properties of pomegranate vinegars are more significant than those of crude pomegranate extracts. According to the results, the vinegars had high levels of total phenolics and flavonoids, demonstrating potent antioxidant capacity and broad-spectrum enzyme-inhibitory activity. Compared to control cholinesterases, elastase, urease, and tyrosinase inhibitory results were in the ranges of 92.47-96.53% in AChE, 87.41-94.35% in BChE, 91.15-98.86%, 89.68-97.5% and 93.35-98.86%, respectively for the vinegars investigated and one of the vinegars (vinegar 1) was the best type of over commercial groups. These observations imply that

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fermentation not only maintains but also can even augment the bioactive profile of pomegranate, forming metabolites that are potentially more bioactive and health-rewarding.

In conclusion, pomegranate vinegar is a promising new functional food that may play essential roles in oxidative stress, neurodegenerative diseases, anti-aging, gastrointestinal disorders, and diabetes. Further *in vivo* and clinical studies are needed to verify these versatile effects and clarify the mechanisms underlying the health benefits of pomegranate vinegars.

Conflict of Interest

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

ORCID

Güleyfe Beyza Yıldırım: [0000-0001-8702-1901](https://orcid.org/0000-0001-8702-1901)

Çağlayan Ünsal Gürer: [0000-0002-8244-4152](https://orcid.org/0000-0002-8244-4152)

İsmail Yener: [0000-0002-0988-9462](https://orcid.org/0000-0002-0988-9462)

Mehmet Akdeniz: [0000-0002-4435-4826](https://orcid.org/0000-0002-4435-4826)

Serkan Yigitkan: [0000-0002-6202-1515](https://orcid.org/0000-0002-6202-1515)

Mustafa Abdullah Yılmaz: [0000-0002-4090-7227](https://orcid.org/0000-0002-4090-7227)

Suzan Tireki: [0000-0002-7424-7378](https://orcid.org/0000-0002-7424-7378)

Abdulselam Ertas: [0000-0002-2193-8386](https://orcid.org/0000-0002-2193-8386)

Aslı Barla Demirköz: [0000-0002-3261-0212](https://orcid.org/0000-0002-3261-0212)

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