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Ultrasound-assisted enhancement of bioactive compounds in hawthorn vinegar: A functional approach to anticancer and antidiabetic effects

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ABSTRACT

In this study, the effects of ultrasound treatment on bioactive components and functional properties of hawthorn vinegar (*Crataegus tanacetifolia*) were investigated. Parameters such as total phenolic compound (TPC), total flavonoid content (TFC), ascorbic acid (AA), DPPH radical scavenging activity and CUPRAC reducing capacity were optimised by surface response method (RSM) and 14 min duration and 61.40 % amplitude were determined as the most suitable treatment conditions. The results showed that ultrasound treatment improved the antioxidant properties of hawthorn vinegar by increasing TPC, TFC, DPPH and CUPRAC values. In addition, it was observed that hawthorn vinegar samples exhibited anticancer effects in cell culture experiments. In experiments on A549 (lung), MCF-7 (breast) and HT-29 (colon) cancer cell lines, ultrasound-treated vinegar increased apoptotic effects, suppressed cell migration and reduced necrosis rates in some cell lines. In particular, ultrasound treatment of vinegar resulted in a reduction in the expression of anti-apoptotic genes (BCL-2 and XIAP) and an enhancement in the expression of pro-apoptotic genes (BAX). These findings suggest that ultrasound technology preserves and enhances the bioactive components of hawthorn vinegar, improves its anticancer properties and increases its potential for use as a functional food product.

1. Introduction

There are various types of vinegar on the market, which has been recognised as a disease healer since ancient times. It is used in foods, especially as a spice and salad dressing [1]. There are more than 1000 varieties of hawthorn worldwide, belonging to the *Rosaceae* family [2]. Hawthorn (*Crataegus spp)* is widely distributed in temperate and subtropical regions and is an important herbal resource due to its rich bioactive compounds and multiple health benefits [3]. Hawthorn has remarkable nutritional and functional value as it is rich in bioactive compounds such as vitamin C, fibre, flavonoids and phenolic acids [4,5]. Hawthorn berry exhibits a wide range of biological activities, such as anti-inflammatory, antibacterial, antioxidant, immunomodulatory, and anticancer effects [6]. This fruit, which has been used in traditional

Abbreviations: C-HV, Untreated hawthorn vinegar; P-HV, Thermally pasteurised hawthorn vinegar; U-HV, Ultrasound-treated hawthorn vinegar; RSM, Response surface methodology; TPC, Total phenolic content; TFC, Total flavonoid content; DPPH, 2,2-diphenyl-1-picrylhydrazyl: radical scavenging activity; CUPRAC, Cupric reducing antioxidant capacity; AA, Ascorbic acid; df, Degrees of freedom; R^2, Coefficient of determination; A549, Lung cancer cell lines; MCF-7, Breast cancer cell lines; HT-29, Colon cancer cell lines; BCL-2, Anti-apoptotic genes; XIAP, Anti-apoptotic genes (XIAP); BAX, Pro-apoptotic genes.

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medicine for generations, has been proven to contain antidiabetic, anticarcinogenic, antioxidant, antibacterial and anti-inflammatory properties [2,7].

Vinegar is made by fermenting sugar- and starch-rich fruits or grains, such as apples, grapes and rice, and has been used for centuries as an acidifier, seasoning and sweetener [8]. Hawthorn vinegar is rich in phenolic compounds and thanks to these components they exert a cardioprotective effect [9]. Various studies have detailed the basic constituents and bioactive contents of hawthorn vinegar. Hawthorn vinegar fermented by traditional methods has been found to contain high levels of phenolic compounds and strong antioxidant capacity [10]. Hawthorn vinegar is rich in gallic acid, caffeic acid and catechin, making it more potent than other fruit vinegars. Other phenolic compounds present include epicatechin, vanillic acid and ellagic acid [11].

Ultrasound treatment is an innovative technology that supports environmental sustainability in the food industry and is attracting increasing interest. It is the result of mechanical waves resonating with solids, liquids and gases. The food industry uses this non-thermal technology in a wide range of applications to improve product quality and optimise processing processes [12]. This technology is a non-thermal alternative to thermal processing in the food industry that is generally applicable to many food products, fruit juices and other products such as vinegar [13–16]. In recent years, several foodstuffs have been subjected to scientific investigation, including strawberry vinegar [15], gilaburu vinegar [14], organic cherry laurel (*Prunus Laurocerasus*) vinegar [16], blackthorn vinegar [17], Blackthorn Vinegar [17], uruset apple vinegar [18] have been produced.

This study aims to investigate the potential of ultrasound technology to preserve and enhance the bioactive components of hawthorn vinegar. At the same time, the effects of this technology on the anticancer and antidiabetic properties of hawthorn vinegar were studied in detail. The study evaluated and compared the effects of ultrasound treatment on biologically essential parameters such as phenolic compounds, flavonoids, and antioxidant activities with conventional thermal pasteurization methods. The findings aim to contribute to the development of hawthorn vinegar as a functional food product and its more practical use in the fight against chronic diseases.

2. Material and methods

2.1. Materials

Samples of hawthorn fruit (*Crataegus tanacetifolia*) were collected from a commercial company in Bursa, Türkiye. This company produces unprocessed hawthorn vinegar using traditional methods [19]. The vinegars obtained by Yıkmış were processed by 3 methods [18]. The acetic acid content of the fermenting hawthorn vinegar was approximately 4 %. The first method refers to the so-called untreated vinegar (C-HV). The second method refers to the vinegar produced by thermal pasteurisation (P-HV). The third method (U-HV) refers to vinegar, which has been treated using the reaction surface method and the application of ultrasound.

2.2. Methods

2.2.1. Ultrasound processing

Samples of hawthorn vinegar were subjected to a series of ultrasound parameters. The process was conducted on a solution of 100 mL of hawthorn vinegar using the Hielscher Ultrasonics UP200St apparatus, manufactured in Berlin, Germany.

The frequency was set at 26 kHz, and the power output was 200 W. Amplitudes of 40 %, 55 %, 70 %, 85 %, and 100 % (in steps of 5 %) and treatment times of 2, 5, 6, 8, 11 and 14 min (in steps of 1 min) were used in continuous mode. An ice water bath was utilized to ensure the process did not overheat. After the ultrasound treatment, the samples were rapidly cooled in an ice bath and stored at −18 ± 1°C for subsequent analysis.

2.2.2. Thermal pasteurization

The samples of Hawthorn vinegar were transferred to 100 mL glass bottles and subjected to pasteurization at 85 ± 1 °C for two minutes, utilizing a water bath system (Wisd model WUC-D06H, Daihan, Wonju, Korea). Upon completion of the pasteurization process, the samples were allowed to cool to 20 ± 1 °C at room temperature and subsequently stored at −20 ± 1 °C until the commencement of an additional phase of analysis.

2.2.3. Ultrasound modeling procedure for RSM

TPC (mg GAE/100 mL), TFC (mg CE/100 mL), AA (mg/100 mL), DPPH (% inhibition), and CUPRAC (% inhibition) values of hawthorn vinegar were optimized by RSM using coded parameters time (X_1: 2–14 min) and amplitude (X_2: 40–100 %). A central composite design (CCD) was used with 13 experimental studies with two selected variables at five levels (−1.41 (very low), −1 (low), 0 (medium), +1 (high), +1.41 (very high)). Minitab software (version 19, Minitab Inc., State College, PA, USA) was applied to optimize the ultrasound treatment. Each experiment was performed with three replications.

The equation models were constructed using the quadratic polynomial formula:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

The following formula definition is provided:

The linear equation coefficient (βi) for the first order.

The quadratic equation coefficient (βii).

The coefficient for the interaction between two factors (βij).

2.2.4. Total phenolic compounds (TPC)

TPC was quantified by the Folin-Ciocalteau method [20]. Total phenolic content (TPC) analysis of hawthorn vinegar samples was performed as follows: A 2 mL aliquot of each sample was mixed with an 8 mL aliquot of a 80 % methanol solution and then centrifuged at 4000 rpm for 20 min using a 5-fold dilution. Following centrifugation, 100 µL of Folin-Ciocalteu reagent and 1500 µL of deionized water were introduced to 50 µL of supernatant solution. The mixture was subsequently allowed to stand for 10 min. At the end of the waiting period, 50 µL of 20 % sodium carbonate solution (Na_2CO_3) was added, and the mixture was allowed to react for 2 h in the dark. The absorbance of the samples was measured at 765 nm, and the results were expressed as mg of gallic acid equivalent per 100 mL sample.

2.2.5. Total flavonoid content (TFC)

In this study, the colorimetric method described by Zhishen et al. was used to determine the total flavonoid content (TFC) [21]. To determine the total flavonoid content, 4 mL of distilled water and 0.3 mL of 5 % sodium nitrite ($NaNO_2$) were added to each 1 mL of the diluted hawthorn vinegar sample. The mixture was incubated for 5 min. Then 0.3 mL of 10 % aluminum chloride ($AlCl_3$) was added and permitted to remain undisturbed for 6 min. Then, 2 mL of 1 M sodium hydroxide (NaOH) is added, and the final volume of the mixture is adjusted to 10 mL with distilled water. The analysis was repeated three times, and the absorbance of the samples was measured using a UV–VIS spectrophotometer at 510 nm. The results are expressed as catechin equivalents (mg CE/L).

2.2.6. Contains ascorbic acid

The ascorbic acid content was determined using the method described by Ordóñez-Santos and Vázquez-Riascos (2010) [22]. 0.2 g oxalic acid ($C_2H_2O_4$) was added to 30 ml of hawthorn vinegar. A 10-ml

solution was titrated with 2,6-dichloroindophenol (DPIP) reagent until a stable dark purple colour was attained. The concentration of ascorbic acid was calculated according to Equation (2).

$$Ascorbicacid(mg/100ml) = \mathrm{MVC} \times \mathrm{MDPIP} \times \mathrm{VDPIP} / 10 \times \mathrm{VS} \quad (2)$$

M_{VC} = molar mass (g/mol) of ascorbic acid, CDPIP = molar concentration (mol/L) of 2,6-dichloroindophenol (DPIP), VS = sample volume (l), VDPIP = volume of DPIP (l).

2.2.7. Antioxidant activity (DPPH and CUPRAC)

With some modifications, the DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used to evaluate the antioxidant activity [23]. A solution of 0.1 mL hawthorn vinegar was added to a solution of 2.9 mL of 0.1 mM DPPH (dissolved in ethanol). The resulting solution was then shaken thoroughly and left to react for 30 min at room temperature without light. Following the incubation period, the absorbance was determined using a UV–VIS spectrophotometer (SP-UV/VIS-300SRB, Australia) at a wavelength of 517 nm. Subsequently, the efficacy of the DPPH radical quenching system was determined by employing the methodology delineated in Equation (3).

$$DPPHradicalscavengingactivity(\%) = (A_0 - A_1)/A_0 x100 \quad (3)$$

A0 is the absorbance of the control sample, while A1 is the absorbance measured for hawthorn vinegar.

The samples' antioxidant capacity was determined using the Cu(II) ion reduction antioxidant capacity (CUPRAC) method [24].

2.2.8. Analysis of phenolic compounds

In a study conducted by Portu et al. (2017), the authors utilized a C-18 (250 × 4.6 mm; 5 μm packing; Agilent) ACE genix column for chromatographic analysis [25]. Polyphenols were subjected to analysis on an Agilent 1260 chromatograph equipped with a diode-array detector (DAD). A flow volume of 0.80 mL/min was employed. The temperature of the column was maintained at 30 °C. Eluents A and B were used for gradient elution. Solution A was water with 0.1 % phosphoric acid, while solution B was acetonitrile. The gradient used was as follows: 17 % B (0 min), 15 % (7 min), 20 % (20 min), 24 % (25 min), 30 % (28 min), 40 % (30 min), 50 % (32 min), 70 % (36 min) and 17 % (40 min). A volume of 10 μL was used for the injection in the phenolic analysis. The study used a UV–Vis spectrophotometer at 280, 320, and 360 nm wavelengths. The results are expressed as micrograms per milliliter of sample.

2.2.9. Antidiabetic activity

A modified method was used to investigate the antidiabetic activity of hawthorn vinegar (α-glucosidase and α-amylase) [26]. For antidiabetic analyses, acarbose was used as a positive control. Absorbance measurements were performed using a UV–VIS spectrophotometer (Spectrum Instrument, SP-UV/VIS-300SRB, Australia).

2.2.10. Cell culture and MTT assay

For the present study, A549, MCF-7, and HT-29 cell lines were commercially obtained from ATCC and maintained under standard culturing conditions as previously described [17]. For the MTT cell viability assay, cells were seeded to 96-well plates and allowed to stand overnight in the CO_2 incubator. Following overnight incubation, cells were incubated with varying concentrations of the hawthorn vinegar for 24 h. MTT assay was carried out as previously mentioned [14].

2.2.11. Annexin V/ PI double staining

For the apoptosis analysis, cells were seeded into 6-well plates at a concentration of 5x105 and incubated overnight. Following incubation, cells were treated with hawthorn vinegar (25 % concentrated) for 24 h. Following treatments, cells were detached using trypsin-EDTA solutions and subjected to Annexin V/ PI staining. Live, early/late apoptotic, and necrotic cell populations were given as percentages by adjusting the gate according to the cells to which no dye was added.

2.2.12. DNA fragmentation assay

For the DNA fragmentation assay, lung, and colon cancer cells were seeded into 12-well plates 5x105 cells/ml, and following overnight incubation, they were subjected to hawthorn vinegar treatment. Following 24 h of overnight incubation, cells were collected, and DNA was isolated using a GeneAll DNA isolation kit (GeneAll Biotechnology, Korea). The isolated DNA samples were measured spectrophotometrically and run on a 1 % agarose gel. Then, images of banding patterns were obtained using a UV transilluminator.

2.2.13. Wound healing experiments

Cells were seeded into 6-well plates for wound healing experiments and allowed to reach full confluency. The well with cell monolayer was scratched with a fine pipette tip to create a scratch and washed twice with PBS to eliminate floating cells. Afterward, Day 0 images were taken, and cells were exposed to 25 % vinegar samples. Images were taken and recorded every 24 h. Analysis was performed by measuring the intervals from the recorded images with the Image J program.

2.2.14. RNA isolation and Real-Time PCR

Like the DNA fragmentation assay, cells were seeded into 12-well plates and subjected to hawthorn vinegar treatments. Following treatments, total RNA was isolated using TriReagent (Nucleogene, Türkiye) following the recommendations of the manufacturer. Spectrophotometric measurement was performed to determine the concentrations of the isolated RNA samples, and the amount to be used in cDNA synthesis was calculated accordingly. cDNA synthesis from RNA was performed using the Nucleogene cDNA synthesis kit (Nucleogene, Türkiye). Afterwards, Real-Time PCR experiments were performed with the Nucleogene SYBR green kit (Nucleogene, Türkiye) using custom-designed gene expression primers (Table 1). The thermal cycling program comprised 10 min at 95 °C, 40 cycles of 20 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. After the reactions, Ct values were determined at an appropriate threshold value and relative gene expression levels were calculated with the $2^{-\Delta Ct}$ formula. GAPDH was used as an internal control gene.

2.2.15. Statistical analysis

All tests were conducted in triplicate, and the resulting data are presented as the mean with standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA), with differences between means assessed using Tukey's mentally significant deviation (HSD) test at a substantial value of $p < 0.05$. Statistical analysis was done using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Three-dimensional RSM plots were generated using Sigma Plot 12.0 statistical analysis software (Systat Software Inc., San Jose, California, USA).

3. Result and discussion

3.1. Bioactive compound optimization

The experimental variables were optimized using the RSM approach, successfully optimizing the experimental parameters. The experimental

Table 1
Primer sequences.

Primer name	Sequences (5′ – 3′)
BAX-Fw	GTCGCCCTTTTCTACTTTGCC
BAX-Rw	TGGTCACGGTCCAACCACC
BCL2-Fw	ATAACGGAGGCTGGGATGC
BCL2-Rw	TCACTTGTGGCCCAGATAGG
XIAP- Fw	CGAGGAACCCTGCCATGTAT
XIAP-Rw	TTCTGACCAGGCACGATCAC
GAPDH-Fw	GTTCGTCATGGGTGTGAACC
GAPDH-Rw	CATGGACTGTGGTCATGAGT

design was devised to investigate the linear interaction and quadratic effects of ultrasound-assisted parameters on the production yield of bioactive components in hawthorn vinegar. Table 2 presents the experimental values of different parameter combinations of hawthorn vinegar, with corresponding predicted yields of total phenolic content (mg GAE/100 ml), total flavonoid content (mg CE/100 ml), antioxidant activity (mg/100 ml), and DPPH (% inhibition) and CUPRAC (% inhibition) values following ultrasound treatment. The findings demonstrated that the optimal outcomes were achieved at 14 min with an amplitude of 61.40 %. By the established conditions, the optimal yields for TPC, TFC, AA, DPPH, and CUPRAC were determined to be 116.99 mg GAE/100 mL, 15.89 mg CE/100 mL, 3.97 mg/100 mL, 62.35 % inhibition, and 67.39 % inhibition, respectively. The coefficients of multiple determination and the results of the analyses of variance were used to evaluate the experimental data to ascertain whether the second-order polynomial model and the derived equation could explain the variability of the responses. The regression equation obtained was subjected to analysis using the ANOVA technique. The significance of the coefficient was evaluated using the F-test and p-test at the 95 % confidence level [27]. As indicated by the outcomes of the F-test, the results demonstrated the degree of statistical significance associated with each coefficient variable. Furthermore, the importance of the interaction between the variables was ascertained by examining the corresponding p-values. An increase in the F value, accompanied by a decrease in the p-value, indicates the significance of the estimated model [28]. It is important to note that p-values of less than 0.05, 0.01, and 0.001 indicate significant, extremely significant, and impressively significant findings, respectively. Moreover, if the p-values exceed 0.05, the model terms are deemed statistically insignificant [29]. Table 3 presents the optimization results, related R^2 values, ANOVA results, incompatibility evaluation, and regression coefficients for TPC (mg GAE/100 mL), TFC (mg CE/100 mL), AA (mg/100 mL), DPPH (% inhibition), CUPRAC (% inhibition) contents of hawthorn vinegar.

X_1: time; X_2: amplitude; RSM: Response surface methodology; GAE-gallic acid equivalent; TPC- total phenolic content; TFC- total flavonoid content; AA: Ascorbic acid; DPPH- 2,2-diphenyl-1-picrylhydrazyl: radical scavenging activity; CUPRAC cupric reducing antioxidant capacity; U-HV: ultrasound-treated hawthorn vinegar.

The effects of two independent variables, duration, and amplitude, on the TPC (mg GAE/100 mL) (Equation (4), TFC (mg CE/100 mL) (Equation (5), AA (mg/100 mL) (Equation (6), DPPH (% inhibition) (Equation (7) and CUPRAC (% inhibition) (Equation (8) properties of hawthorn vinegar are given below.

$$TPC(mgGAE/100mL) = 82,296 + 5,002X_1 + 0,3838X_2 + 0,00910X_1X_1 + 0,000441X_2X_2 - 0,07252X_1X_2 \quad (4)$$

$$TFC(mgCE/100mL) = 7,444 + 0,7707X_1 + 0,14285X_2 + 0,00093X_1X_1 - 0,000496X_2X_2 - 0,010962X_1X_2 \quad (5)$$

$$AA(mg/100mL) = 7,163 - 0,4663X_1 - 0,04318X_2 + 0,009966X_1X_1 + 0,000076X_2X_2 + 0,004360X_1X_2 \quad (6)$$

$$DPPH(\%inhibition) = 37,490 + 2,578X_1 + 0,4443X_2 - 0,05675X_1X_1 - 0,002180X_2X_2 - 0,02229X_1X_2 \quad (7)$$

$$CUPRAC(\%inhibition) = 41,461 + 2,6278X_1 + 0,46292X_2 - 0,05334X_1X_1 - 0,002202X_2X_2 - 0,023884X_1X_2 \quad (8)$$

According to the equations, the increase in X_1 (time) and X_2 (amplitude)

Table 2
Ultrasound RSM analysis of dependent and independent variables and TPC, TFC, AA, DPPH, and CUPRAC results.

Run no.	Independent variables		Dependent variables									
	Time (X_1) (min)	Amplitude (X_2) (%)	TPC (mg GAE/100 mL)		TFC (mg CE/100 mL)		AA (mg/100 mL)		DPPH (% inhibition)		CUPRAC (% inhibition)	
			Experimental data	RSM predicted	Experimental data	RSM predicted	Experimental data	RSM predicted	Experimental data	RSM predicted	Experimental data	RSM predicted
1	11 (+1)	55 (−1)	117.02	116.99	15.85	15.76	3.74	3.73	63.40	63.34	68.23	68.26
2	11 (+1)	85 (+1)	106.50	106.42	14.42	14.34	4.18	4.19	60.19	60.16	64.96	65.02
3	8 (0)	70 (0)	111.30	111.31	15.08	15.10	3.86	3.86	62.15	62.42	67.33	67.31
4	2 (−1.41)	70 (0)	111.17	111.21	15.05	15.02	4.25	4.23	59.67	59.72	64.74	64.77
5	8 (0)	70 (0)	111.35	111.31	15.08	15.10	3.86	3.86	62.45	62.42	67.33	67.31
6	8 (0)	70 (0)	111.35	111.31	15.08	15.10	3.86	3.86	62.45	62.42	67.33	67.31
7	8 (0)	70 (0)	111.35	111.31	15.08	15.10	3.86	3.86	62.45	62.42	67.33	67.31
8	14 (+1.41)	70 (0)	112.03	112.06	15.17	15.24	4.20	4.21	61.01	61.03	66.05	66.00
9	5 (−1)	85 (+1)	112.61	112.52	15.18	15.22	3.76	3.81	61.54	61.50	66.57	66.55
10	8 (0)	100 (+1.41)	107.62	107.67	14.22	14.22	4.02	4.00	59.28	59.28	64.26	64.23
11	5 (−1)	55 (−1)	110.11	110.03	14.69	14.66	4.13	4.13	60.84	60.67	65.57	65.5
12	8 (0)	70 (0)	111.04	111.31	15.08	15.10	3.86	3.86	62.45	62.42	67.22	67.31
13	6 (0)	40 (−1.41)	111.26	111.29	14.35	14.39	4.15	4.16	59.77	59.85	64.55	64.57
U-HV (RSM optimization parameters)	14 min	61.40 % amplitude	116.99		15.89		3.97		62.35		67.39	
Experimental values			114.86 ± 0.42		15.32 ± 0.31		4.08 ± 0.18		61.56 ± 1.24		66.94 ± 1.47	
% Difference			1.82 %		0.00 %		2.69 %		1.26 %		0.66 %	

Table 3
A factorial ANOVA was employed as a regressor in the central combination test model.

		TPC (mg GAE/100 mL)		TFC (mg CE/100 mL)		AA (mg/100 mL)		DPPH (% inhibition)		CUPRAC (% inhibition)	
Source	**DF**	**F-Value**	**P-Value**	**F-Value**	**P-Value**	**F-Value**	**P-Value**	**F-Value**	**P-Value**	**F-Value**	**P-Value**
Model	5	968.98	0.000	123.50	0.000	125.96	0.000	242.89	0	1163.85	0.000
Linear	2	1421.11	0.000	69.34	0.000	10.14	0.009	132.07	0	546.39	0.000
X_1	1	36.31	0.001	10.00	0.016	0.51	0.499	75.52	0.000	317.16	0.000
X_2	1	2841.22	0.000	134.38	0.000	20.19	0.003	210.05	0.000	864.79	0.000
Square	2	9.71	0.010	38.57	0.000	153	0.000	260.33	0.000	1171.72	0.000
X_1X_1	1	10.24	0.015	0.44	0.527	305.51	0.000	348.19	0.000	1466.77	0.000
X_2X_2	1	13.37	0.008	70.58	0.000	9.75	0.017	285.18	0.000	1387.63	0.000
2-Way Interaction	1	3103.41	0.000	295.67	0.000	278.88	0.000	256.14	0.000	1402.36	0.000
X_1X_2	1	3103.41	0.000	295.67	0.000	278.88	0.000	256.14	0.000	1402.36	0.000
Error	7										
Lack-of-Fit	3	0.59	0.652	*	*	*	*	0.91	0.512	1.88	0.275
Pure Error	4										
Total	12										
R^2		99.86 %		98.88 %		98.90 %		99.43 %		99.88 %	
Adj. R^2		99.75 %		98.08 %		98.12 %		99.02 %		99.79 %	
Pred. R^2		99.55 %		87.39 %		88.87 %		97.85 %		99.24 %	

variables has a linear positive effect on the TPC, TFC, DPPH, CUPRAC values of hawthorn vinegar. TPC was measured in mg GAE/100 mL and both experimental and predicted values were quite close in each condition. For example, while the experimental TPC value of Run 1 was 117.02 mg GAE/100 mL, the predicted value was 116.99 mg GAE/100 mL. When the amplitude was 55 % and the time was 11 min, the TPC value was observed at the highest level. As the amplitude increased, especially at 85 % amplitude (2nd run), it was observed that TPC decreased (106.50 mg). When the optimization parameters (14 min, 61.40 % amplitude) were examined, the TPC value was estimated as 116.99 mg GAE/100 mL and it is compatible with the experimental data (114.86 mg) with a difference of 1.82 %. This shows that the RSM model can predict the phenolic content with high accuracy (Table 2). According to the ANOVA results, the model is highly significant on total phenolic content (TPC) (F-value: 968.98, P-value: 0.000). The linear effects are also quite strong; F-value is 36.31 and P-value is 0.001 for X_1 (time), F-value is 2841.22 and P-value is 0.000 for X_2 (amplitude). In addition, the interaction effect of X_1 and X_2 is also highly significant (F-value: 3103.41, P-value: 0.000). The R^2 value of 99.86 % of the model indicates that almost all of the variance can be explained, which supports the reliability of the model for phenolic content (Table 3).

X_1: time; X_2: amplitude; DF: degrees of freedom; R^2—coefficient of determination; $p < 0.05$, significant differences; $p < 0.01$, very significant differences: GAE- gallic acid equivalent; TPC- total phenolic content; TFC- total flavonoid content; AA: ascorbic acid; DPPH- 2,2-diphenyl-1-picrylhydrazyl: radical scavenging activity; CUPRAC: cupric reducing antioxidant capacity.

TFC was reported as mg CE/100 mL and again there were small differences between the experimental data and the RSM predictions. Generally, the difference between the predicted and experimental values was less than 1 %. For example, the experimental TFC value of run 1 was 15.85 mg CE/100 mL while the predicted value was 15.76 mg. The flavonoid content showed slight changes as the amplitude and time increased. For example, when the amplitude increased to 85 % in run 2, the TFC decreased slightly to 14.42 mg CE/100 mL. Under the optimization parameters, the flavonoid content was determined as 15.32 mg CE/100 mL experimentally and 15.89 mg CE/100 mL predicted, which is in good agreement with only 0.00 % difference (Table 2). The F-value of the model for the total flavonoid content was calculated as 123.50 and the P-value as 0.000, indicating that the model was highly significant (Table 2). A very strong effect is observed for the amplitude variable (X_2) with an F-value of 134.38 and a P-value of 0.000. The interactions of X_1 and X_2 are also highly significant (F-value: 295.67, P-value: 0.000). The R^2 value for TFC is 98.88 % and the adj. R^2 value is 98.08 %, indicating a high degree of model accuracy and data compatibility. This increase in the bioactive compound content of hawthorn vinegar can be attributed to acoustic and hydro cavitation, where microbubbles are suddenly formed, expanded, and collapsed in the solvent. Ultrasound treatment increases the accessibility of solid particles to the solvent, changes the internal architecture of the plant cell, and intensifies mass transfer rates [30,31]. In their study on strawberry vinegar, Türkol et al (2024) concluded that ultrasound treatment increased the TPC, TFC and DPPH values of strawberry vinegar, similar to our study [15]. Similar to our research, Erdal et al. (2022) reached high bioactive values due to the treatment of gilaburu vinegar with different ultrasound parameters. [32]. These results are consistent with another study that found that ultrasound treatment increased the bioactive components in vinegars [33].

AA content was measured as mg/100 mL, and the predicted values were almost the same as those of the experimental results. According to the equation, AA value is negatively affected by the increase in X_1 parameter while it is positively affected by the increase in X_2 parameter. Especially, ascorbic acid content of the 1st run was measured as 3.74 mg/100 mL and the predicted value was calculated as 3.73 mg. When the amplitude increased to 85 %, the ascorbic acid content showed a slight increase (4.18 mg/100 mL) and this trend continued with the increase in time. While the predicted value in the optimization parameters was 3.97 mg/100 mL, the experimental result was observed as 4.08 mg/100 mL, which shows a difference of only 2.69 %. It was observed that the effect of ultrasound on ascorbic acid was stable according to time and amplitude combinations. The overall F-value of the model for ascorbic acid was found as 125.96 and the P-value as 0.000. (Table 3). For the amplitude variable (X_2), the F-value was calculated as 20.19 and the P-value as 0.003, indicating that the amplitude has a significant effect on the ascorbic acid content. In addition, the interaction effect of time and amplitude is also quite strong (F-value: 278.88, P-value: 0.000). The R^2 value of the model is 98.90 %, indicating that the model can predict the ascorbic acid content with high accuracy.

According to the equations, DPPH (% inhibition) and CUPRAC (% inhibition) values are positively affected by X1 and X2 parameters, and negatively affected by the square effect of X_1 and X_2 and the interaction effect of X_1 and X_2. The 1st run DPPH inhibition value of 63.40 % was estimated as 63.34 % by RSM, the difference is quite small. When the amplitude increased to 85 % (2nd run), the inhibition level decreased to 60.19 %. Small increases in inhibition levels were generally observed in conditions where time increased. Only 1.26 % difference was found between the estimated value of 62.35 % and the experimental value of 61.56 % in the optimization parameters, which shows that the

prediction of DPPH activity was quite successful. In addition, when the ANOVA results in Table 3 are examined, the results for DPPH inhibition show that the model is very strong. The overall F-value of the model is 242.89 and the P-value is 0.000. The F-value of X_1 (time) variable was calculated as 75.52 and the P-value as 0.000, and the F-value of X_2 (amplitude) variable was calculated as 210.05 and the P-value as 0.000. The interaction between time and amplitude was highly significant (F-value: 256.14, P-value: 0.000). The R2 value was 99.43 %, indicating that the model could accurately explain DPPH activity. CUPRAC value 1st run showed 68.23 % inhibition, while RSM predicted 68.26 %. The differences were relatively slight. As time and amplitude increased, CUPRAC values generally decreased. For example, CUPRAC was measured as 64.26 % and predicted as 64.23 % at 100 % amplitude and 8 min. In the optimization parameters, the expected value for CUPRAC is 67.39 % and the experimental value is 66.94 %, which shows a very close fit with a difference of 0.66 %. For CUPRAC inhibition, the model's overall F-value was 1163.85 and the P-value was 0.000, indicating that the model is strong and significant on CUPRAC. For the amplitude variable (X_2), the F-value was calculated as 864.79 and the P-value was 0.000, indicating that the amplitude strongly affects CUPRAC. The interaction of time and amplitude was also highly significant (F-value: 1402.36, P-value: 0.000). The R^2 value for CUPRAC was 99.88 %, indicating that almost all of the variance was explained by the model (Table 3).

As a result, very low differences were observed between the estimated and experimental values for the bioactive analyses (TPC, TFC, AA, DPPH, and CUPRAC) in the table. This shows that the surface response method (RSM) performed on the bioactive contents of ultrasound-treated hawthorn vinegar gave accurate results and was consistent with the experimental data. According to ANOVA analyses, the model used for each bioactive component (TPC, TFC, AA, DPPH, and CUPRAC) was statistically significant. Since all P-values were below 0.05 and at very low levels ($P < 0.001$) for most variables, the reliability of the model and the consistency of the results were relatively high.

In Fig. 1A, TPC (mg GAE/100 mL) increases with increasing amplitude and duration, suggesting that ultrasound positively affects the extraction of phenolic compounds. In Fig. 1B, TFC (mg CE/100 mL), similarly increases with increasing amplitude and duration, indicating that flavonoids are released more and their health benefits increase. While the AA (mg/100 mL), content reached the highest values at the optimal amplitude and duration in Fig. 1C, DPPH (% inhibition) free radical scavenging activity increased with increasing amplitude and duration in Fig. 1D, indicating that ultrasound increases antioxidant activity. In Fig. 1E, CUPRAC (% inhibition) increased with increasing amplitude and time, indicating that total antioxidant capacity can be optimized.

These results indicate that ultrasound treatment is an effective method of increasing the phenolic, flavonoid, and antioxidant

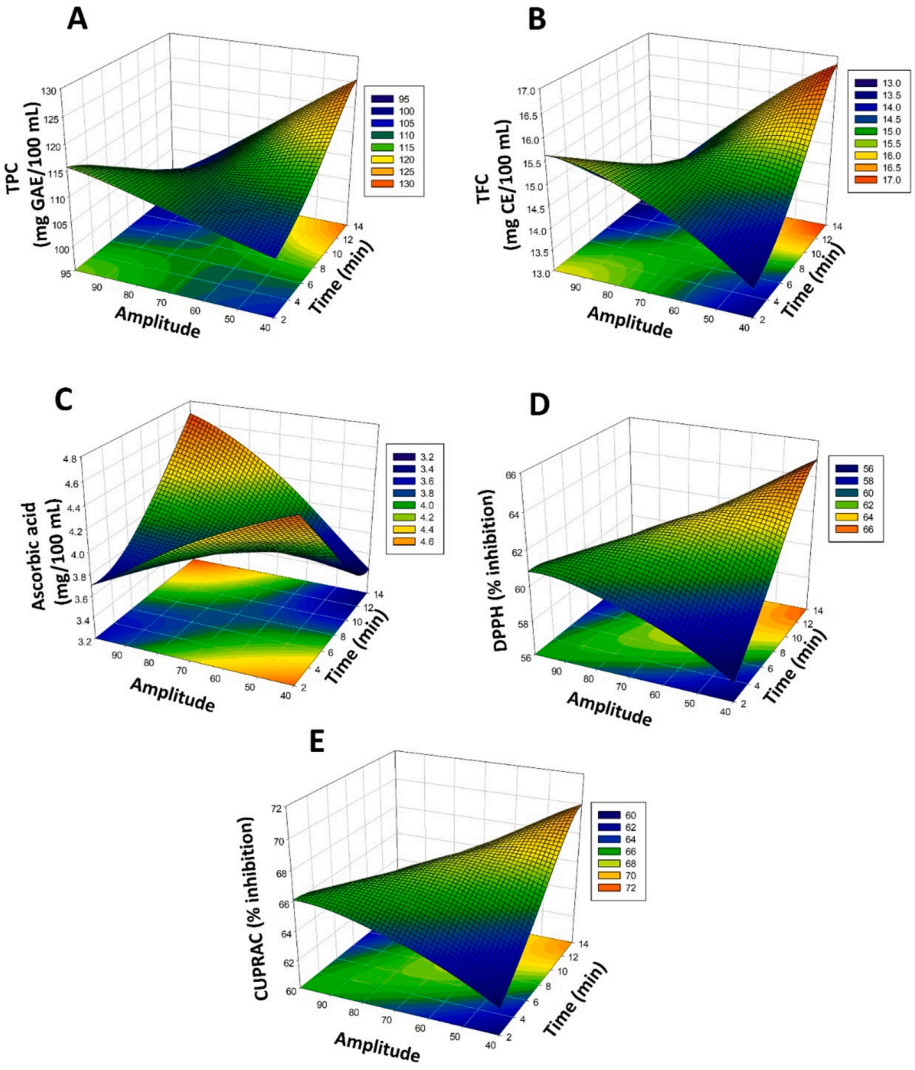

Fig. 1. Response surface plots (3D) for TPC (mg GAE/100 mL), TFC (mg CE/100 mL), AA (mg/100 mL), DPPH (% inhibition) and CUPRAC (% inhibition) amounts of ultrasound treated hawthorn vinegar as a function of the significant factors for RSM.

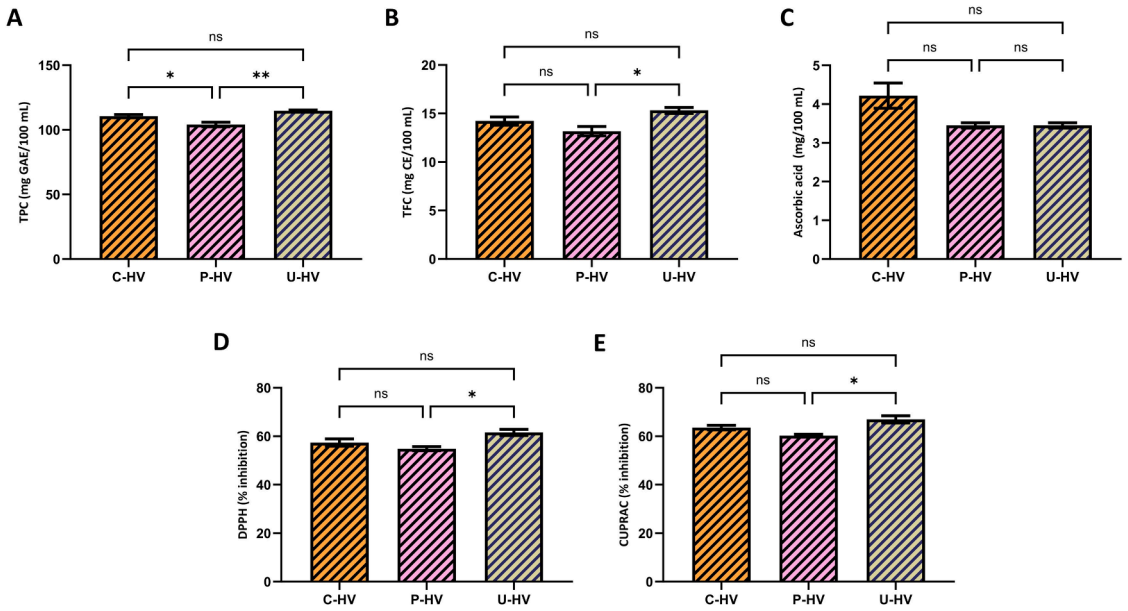

Fig. 2. Control hawthorn vinegar (C-HV), thermal pasteurized hawthorn vinegar (P-HV), ultrasound treated hawthorn vinegar (U-HV) samples were tested for TPC, TFC, AA, DPPH and CUPRAC values. Letters atop bars indicate statistically significant differences (ns: no significant; *p < 0.05; **p < 0.01, (n = 3 ± SD).

components of hawthorn vinegar and that the treatment parameters should be carefully adjusted.

3.2. Bioactive components

C-HV, P-HV, and UT-HV samples were compared in terms of their bioactive compounds. Fig. 2 shows the TPC, TFC, AA, DPPH, and CUPRAC values of bioactive compounds analysed in hawthorn vinegar.

Phenolic compounds are structures having an aromatic ring attached to one or more hydroxyl groups [34]. Ultrasound-treated vinegar (U-HV) had the highest value for TPC (114.86 ± 0.42 mg GAE/100 mL). The control vinegar (C-HV) showed a phenolic content of 110.58 ± 1.22 mg GAE/100 mL, while the thermally pasteurised vinegar (P-HV) showed the lowest value with 104.22 ± 1.73 mg GAE/100 mL. According to statistical analyses, ultrasound treatment significantly increased the phenolic content ($p < 0.05$). The component level of phenolic acids varies at different stages of fermentation in vinegar production[35]. The preservation of phenolic compounds plays a critical role in enhancing antioxidant capacity and maintaining health benefits. While the thermal pasteurization process causes losses in phenolic compounds, ultrasound treatment prevents these losses and helps to obtain higher phenolic content—Türkol ve ark. (2024) determined the amount of TPC in strawberry vinegar as 75.78 ± 2.25 mg GAE/100 mL in the control sample (C-SV), 72.97 ± 1.83 mg GAE/100 mL in the thermal pasteurization treated sample (P-SV), and 81.07 ± 1.38 mg GAE/100 mL in ultrasound treated sample (UT-SV). Similar to our study, it was concluded that thermal pasteurization decreased the TPC content of strawberry vinegar while increasing the TPC content in ultrasound-treated samples [15]. According to another study on purple onion vinegar, increases in bioactive components were detected due to ultrasound treatments compared to untreated purple onion vinegar. In contrast, decreases were detected due to thermal pasteurization [36]. Compared to thermal pasteurization, ultrasound treatment applied on trust apple cider vinegar showed similar results to our study and increased the bioactive components of the vinegar 5. The increases in bioactive components observed in hawthorn vinegar may be associated with the reactions caused by ultrasound treatment due to cavitation [32].

Numerous plant-derived bioactive compounds show considerable promise in preventing chronic diseases, and flavonoids constitute an important class of naturally occurring polyphenolic compounds [37]. Ultrasound-treated vinegar (U-HV) showed the highest value for flavonoid content (15.32 ± 0.31 mg CE/100 mL), while control vinegar (C-HV) had 14.22 ± 0.42 mg CE/100 mL and thermally pasteurized vinegar (P-HV) had 13.18 ± 0.48 mg CE/100 mL. Statistical analyses showed that ultrasound treatment made a significant difference on flavonoid content ($p < 0.01$). This indicates that ultrasound treatment effectively preserves and increases flavonoid compounds, while thermal treatment may degrade some of the flavonoids. A study concluded that the amount of TFC in strawberry vinegar decreased by 4.23 % with a thermal pasteurization process similar to our study. In comparison, it increased by 10.81 % with ultrasound treatment [15]. Bioactive compounds were increased due to ultrasound treatment of organic cranberry (*Cornus mas* L.) vinegar. It was reported that total phenolic content increased by 4.6 % with ultrasound treatment [38]. Bioactive components in organic laurel vinegar (*Prunus laurocerasus*) showed results consistent with our study as a result of ultrasound treatment [39].

Ascorbic acid (or vitamin C) is a water-soluble vitamin. The human body cannot produce this vitamin, so providing the appropriate amount of ascorbic acid through our diet is essential. Ascorbic acid content is also influenced by growing, weather or storage conditions and other factors such as pH, temperature or light exposure 10. Control vinegar (C-HV) had the highest value with an ascorbic acid content of 4.22 ± 0.33 mg/100 mL. In comparison, ultrasound-treated vinegar (U-HV) showed a similar performance with 4.08 ± 0.18 mg/100 mL, showing some decrease. However, thermally pasteurized vinegar (P-HV) had the lowest ascorbic acid content with 3.45 ± 0.07 mg/100 mL. During thermal processing, sensitive components such as ascorbic acid are adversely affected by high temperatures. Ultrasound treatment is a more effective for preserving ascorbic acid than thermal treatments because the temperature increase during this process is minimal, and the loss of ascorbic acid is minimized. Wang et al. (2019) observed that ultrasound treatment reduced the ascorbic acid value of kiwi juice compared to the control sample [40]. Ascorbic acid is sensitive to high temperature, light and pressure. This may be due to the breakdown of ascorbic acid by aerobic or anaerobic pathways [41]. The effect of ultrasonic waves on ascorbic acid may be unclear. In another study on mulberry juice, after 60 min of ultrasonic treatment, they observed a significantly increased ascorbic acid content in mulberry juice by about 50 % compared to pressed juice alone. They reported that the ascorbic acid content decreased when the ultrasonic time was extended [42].

Ultrasound-treated vinegar (U-HV) had the highest free radical scavenging capacity with 61.56 ± 1.24 % DPPH inhibition. Control vinegar (C-HV) showed 57.39 ± 1.48 %, and thermal pasteurized vinegar (P-HV) showed 54.86 ± 0.82 % DPPH inhibition. These differences were statistically significant ($p < 0.01$). As with our study, the findings of Yıkmış et al. (2024) indicated that thermal pasteurization resulted in a 5.97 % reduction in antioxidant activity in gilaburu juice. Conversely, ultrasound treatment was observed to enhance this activity by 4.59 %. Thermal pasteurization resulted in a markedly diminished concentration of DPPH, as compared to the control group. Conversely, ultrasound treatment yielded a notable elevation in the levels of DPPH, in contrast to the effects of thermal pasteurization [43]. Ultrasound treatment increases phenolic and flavonoid compounds' free radical scavenging capacity, resulting in a more potent antioxidant effect. This finding supports increasing antioxidant activity in foods and providing higher health benefits.

CUPRAC inhibition measures the antioxidant capacity of a substance against copper ions and is another crucial indicator of antioxidant activity. Ultrasound-treated vinegar (U-HV) showed the highest value with 66.94 ± 1.47 % CUPRAC inhibition. Control vinegar (C-HV) and thermal pasteurized vinegar (P-HV) showed 63.55 ± 0.92 % and 60.22 ± 0.61 % CUPRAC inhibition, respectively. These differences were statistically significant ($p < 0.01$). The application of ultrasound treatment has been demonstrated to enhance the antioxidant capacity of a given substance, thereby providing a more robust effect of phenolic compounds and other antioxidants against copper ions. Thermal treatment decreases this capacity. Similar to our study, in another study conducted on black vinegar, it was concluded that ultrasound treatment increased the antioxidant activity while pasteurization reduced the antioxidant activity [17].

These results indicate that ultrasound treatment is the most effective method for preserving phenolic compounds, increasing flavonoid content and improving antioxidant capacity. Ultrasound treatment contributes to the preservation of higher levels of phenolic and flavonoid compounds while increasing DPPH and CUPRAC. Even for sensitive components such as ascorbic acid, ultrasound treatment protects without significant loss in contrast to thermal pasteurization. Thermal pasteurization gave the lowest results for all bioactive components and is not considered suitable for preserving bioactive components in foods. Ultrasound treatment is coming to the forefront in the food industry to improve quality and preserve bioactive components.

3.3. Phenolic components

In this study, the effects of different processing methods (control, pasteurization, and ultrasound) on the amounts of phenolic compounds in hawthorn vinegar were investigated in detail. Phenolic compounds are secondary plant metabolites consisting of aromatic rings linked to one or more hydroxyl groups [44]. Hawthorn vinegar samples were analyzed for 13 phenolic compounds and presented in Table 4.

The results revealed that especially ultrasound treatment played an essential role in preserving phenolic compounds and increasing their amounts. The amount of chlorogenic acid reached 4.66 μg/mL in ultrasound-treated hawthorn vinegar (U-HV), 3.89 μg/mL in the control group (C-HV), and only 0.93 μg/mL in pasteurized vinegar (P-HV). Caffeic acid was 1.91 μg/mL in C-HV and 6.91 μg/mL in U-HV. p-Coumaric acid was 0.91 μg/mL in C-HV and 3.81 μg/mL in U-HV. Ultrasound treatment increased the amount of chlorogenic acid, caffeic acid, and p-coumaric acid phenolic compounds in hawthorn vinegar. In contrast to our study, Dadan et al. (2022) found no significant difference in the amounts of chlorogenic acid, caffeic acid, and p-coumaric acid in blue honeysuckle (*Lonicera caerulea* L.) [45]. In our study, hawthorn vinegar was found to be rich in chlorogenic acid and caffeic acid content. Similar results with our study were reported in the study conducted by Özdemir et al. (2021) on hawthorn vinegar [46].

Chlorogenic and caffeic acids in hawthorn vinegar have antimutagenic, carcinogenic, anti-inflammation and antioxidant activities. These phenolic compounds show cardioprotective effects against cardiovascular diseases by inhibiting low-density lipoprotein oxidation and lowering blood pressure [9]. Hawthorn vinegar, which has a positive impact in health thanks to these phenolic compounds, has become rich in health-beneficial compounds thanks to ultrasound treatment in our study. Similarly, the amount of catechin Hydrate was 39.79 μg/mL in ultrasound-treated vinegar, while this value was 10.91 μg/mL in the control group and 13.10 μg/mL in pasteurized vinegar. Similarly, the highest amount of caffeic acid was found in the ultrasound-treated vinegar sample (6.91 μg/mL) and 3.60 μg/mL in the pasteurized vinegar (P-HV). These data indicate that ultrasound treatment significantly affects both the preservation and enhancement of phenolic compounds. According to statistical analyses, these differences are significant at $p < 0.05$ level and confirm that phenolic compounds vary according to the processing method. Similar to our study, Lopez-Martinez et al. (2022) concluded that the amounts of catechin and quercetin were higher after ultrasound treatment of turmeric added mango and carrot beverage than the control. This suggests that phenolic compounds are released from cell wall structures due to ultrasound treatment [47].

Table 4
Properties of phenolic compounds of C-HV, P-HV, and U-HV Samples.

Analyzes		Samples		
		C-HV	P-HV	U-HV
Phenolic compounds (μg/mL)	Chlorogenic Acid	3.89 ± 0.16^{b}	0.93 ± 0.04^{a}	4.66 ± 0.13^{c}
	Catechin Hydrate	10.91 ± 0.45^{a}	13.10 ± 0.57^{a}	39.79 ± 1.15^{b}
	Caffeic Acid	1.91 ± 0.08^{a}	3.60 ± 0.16^{b}	6.91 ± 0.20^{c}
	Vanillin	0.24 ± 0.01^{a}	0.22 ± 0.01^{a}	1.07 ± 0.03^{b}
	p-Coumaric Acid	0.91 ± 0.04^{a}	0.94 ± 0.04^{a}	3.81 ± 0.11^{b}
	Rutin	1.17 ± 0.05^{b}	0.37 ± 0.01^{a}	5.26 ± 0.15^{c}
	t-Ferulic Acid	1.28 ± 0.05^{a}	1.08 ± 0.04^{a}	3.19 ± 0.09^{b}
	Hydroxycinnamic Acid	0.79 ± 0.03^{a}	0.64 ± 0.03^{a}	1.89 ± 0.06^{b}
	Naringin	2.12 ± 0.08^{b}	1.54 ± 0.06^{a}	4.87 ± 0.14^{c}
	Rosmarinic Acid	1.15 ± 0.05^{b}	0.50 ± 0.02^{a}	1.42 ± 0.04^{c}
	Salicylic Acid	0.13 ± 0.01^{a}	0.24 ± 0.01^{a}	2.98 ± 0.08^{b}
	Resveratrol	0.12 ± 0.00^{a}	n.d	0.59 ± 0.02^{b}
	Quercetin	2.75 ± 0.08^{a}	n.d	6.56 ± 0.19^{b}

The results are reported as the mean ± standard deviation ($n = 3$). The values marked with different letters within the same line are significantly different ($p < 0.05$). Control hawthorn vinegar (C-HV), thermal pasteurized hawthorn vinegar (P-HV), ultrasound treated hawthorn vinegar (U-HV) and not detected (n.d).

In a study conducted by Margeon et al. (2020), red grape juice was subjected to thermal pasteurization and ultrasound treatment. The results demonstrated that ultrasound treatment yielded more favorable outcomes in terms of polyphenol content [48]. This finding coincides with our study and indicates that ultrasound treatment may be more effective in preserving or increasing polyphenols. When the status of phenolic compounds in other groups is also evaluated, it is seen that ultrasound treatment provides significantly higher values, especially in compounds such as vanillin, rutin, naringin, and quercetin. Quercetin concentration was determined as 2.75 μg/mL in C-HV and 6.56 μg/mL in U-HV. Erdal et al. (2022) found that p-coumaric acid and quercetin levels increased significantly after ultrasound treatment, similar to the present study [32].

Rutin content in the ultrasound-treated vinegar sample was 5.26 μg/mL, while this value was 1.17 μg/mL in the control group and only 0.37 μg/mL in the pasteurized vinegar sample. Similarly, Naringin content increased to 4.87 μg/mL in the ultrasound treatment, while it remained at 2.12 μg/mL and 1.54 μg/mL in the control and pasteurized vinegar samples, respectively. These differences were statistically significant and confirmed at the $p < 0.05$ level. In contrast, pasteurization decreased the number of phenolic compounds in general.

In conclusion, ultrasound treatment is an efficacious method for increasing the bioavailability of phenolic compounds and maintaining their antioxidant properties. In light of these data, using ultrasound technology to preserve and increase phenolic compounds in hawthorn vinegar production can be considered a more advantageous option compared to traditional methods such as pasteurization.

3.4. Antidiabetic activity

In this study, the inhibitory activities of hawthorn vinegar (C-HV), pasteurized hawthorn vinegar (P-HV), and ultrasound-treated hawthorn vinegar (U-HV) against α-glucosidase and α-amylase enzymes were compared. Antidiabetic inhibitory activities are shown in Fig. 3. In terms of α-glucosidase inhibitory activity, the inhibitory activity of C-HV was 37.55 ± 0.08 %, P-HV 34.46 ± 1.21 % and U-HV 39.88 ± 1.4 %. Statistical analyses showed no significant difference (ns: no significant) between C-HV and P-HV. However, ultrasound-treated hawthorn vinegar (U-HV) exhibited statistically significantly higher inhibitory activity compared to both C-HV and P-HV (*$p < 0.05$). This finding reveals that ultrasound treatment significantly increases α-glucosidase enzyme inhibition. According to the results of α-amylase inhibitory activity, the inhibitory activity of C-HV was 35.15 ± 0.68 %, P-HV was 32.12 ± 1.14 % and U-HV was 36.42 ± 1.05 %. The inhibitory activity of P-HV was significantly lower than that of C-HV (*$p < 0.05$), indicating that pasteurization may reduce α-amylase inhibitory activity. In contrast, U-HV provided significantly higher inhibition of both α-glucosidase and α-amylase compared to the other two groups (**$p < 0.01$). These findings suggest that ultrasound treatment may enhance the functional activity of hawthorn vinegar and increase its inhibitory capacity on glucose and starch digestion. α-amylase and α-glucosidase are the primary enzymes responsible for catalyzing the last stage of the digestive system's breakdown of carbohydrates. Inhibition of α-glucosidase and α-amylase, resulting in decreased glucose release and absorption, is a significant approach to lowering blood glucose levels and hyperglycemia in controlling diabetes [49,50]. A study revealed that hawthorn polyphenols predominantly consist of quercetin (74.58 %) and hyperoside (9.58 %), which are significant contributors to biological activity in inhibiting α-glucosidase [51]. Polysaccharides, a significant component of hawthorn fruit, have been found to have substantial α-amylase inhibitory action [52]. Ultrasound technology is used in several food sectors for applications, including microbial inactivation, enzyme inactivation, extraction, emulsification, and fractionation in the dairy industry, as well as thermosonication in fruit juices [53].

Using ultrasound treatment represents an efficacious methodology for extracting aromatic compounds and other phytochemicals from plant matrix structures. Furthermore, this technique is applicable in the separation of diverse chemical components. Treatment with ultrasound is regarded as one of the least intrusive, most energy-efficient, and, crucially, most ecologically sustainable techniques. The process is characterized by a rapid and uncomplicated methodology that allows for the retrieval of a considerable quantity of sample material within a relatively short timeframe while ensuring the preservation of the most vulnerable phytochemicals, including antioxidants [54–56]. Previous studies indicate that ultrasound-treated hawthorn vinegar positively influences intestinal health, enhances immunity, and promotes general well-being in female Wistar albino rats [57]. Another study showed that treating rats with 1 mL/kg of ultrasound-treated hawthorn vinegar greatly enhanced the liver's antioxidant capacity [6]. Aierken et al. also discovered that hawthorn extracts in diabetes enhance plasma release of insulin and decrease blood glucose in high-fat diet-induced dyslipidemia mice [58]. Xin et al. showed that polyphenols, specifically quercetin and hyperoside, in hawthorn fruits, inhibited alpha-glucosidase activity in diabetic rats [51]. Mecheri et al. demonstrated that whole hawthorn fruit exhibited resistance to α-amylase and α-glucosidase, indicating its potential as a hypoglycemic agent [59]. Our findings indicated that U-HV showed an antidiabetic effect by providing greater inhibition of α-glucosidase and α-amylase than C-HV and P-HV. The ultrasonic process of hawthorn vinegar enhances antidiabetic effect by potentially protecting or activating the bioactive components present in hawthorn vinegar. The findings indicate that processing methods are essential for improving the antidiabetic potential of natural products like hawthorn vinegar. The significant inhibitory actions seen in ultrasound-treated hawthorn vinegar suggest that this technology might serve as a helpful approach in producing functional foods and alternative treatments. The general physiological mechanisms can be effectively well, leading to improvements in health status.

3.5. Anticancer activity

In this study, the cytotoxic effect of vinegar samples obtained from different processes on cancer cells was initially determined through MTT experiments. A549, MCF7, and HT29 cells were treated with vinegar samples at varying concentrations for 24 h, after which cell viability analyses were conducted. After determining the half-maximal inhibitory concentration (IC_{50}), induction of apoptosis, DNA fragmentation assay, wound healing experiments, and expression levels of apoptosis-related

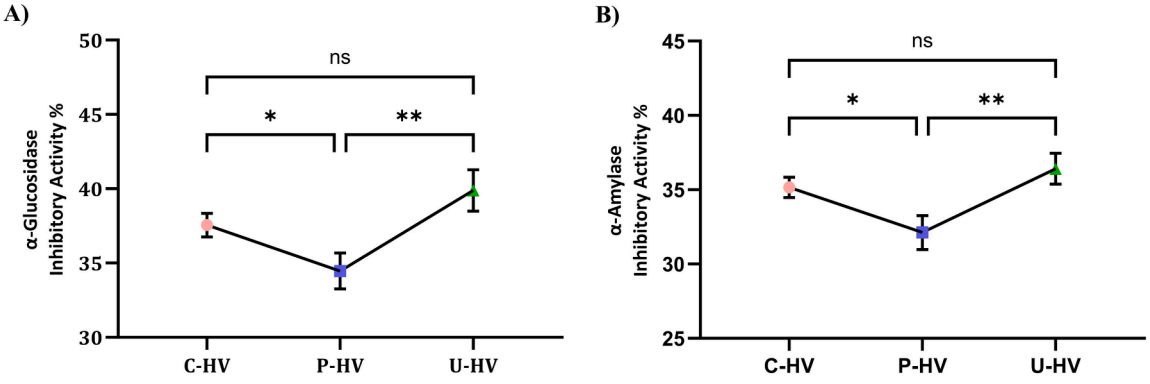

Fig. 3. Antidiabetic inhibitory activities (A and B) of hawthorn vinegar (C-HV), thermal pasteurized hawthorn vinegar (P-HV), ultrasound treated hawthorn vinegar (U-HV). The presence of letters atop bars indicates the existence of statistically significant differences. The following symbols are used to indicate the level of significance: ns (no significant difference), *$p < 0.05$, and **$p < 0.01$. The number of replicates (n) is stated in parentheses, followed by the standard deviation (SD).

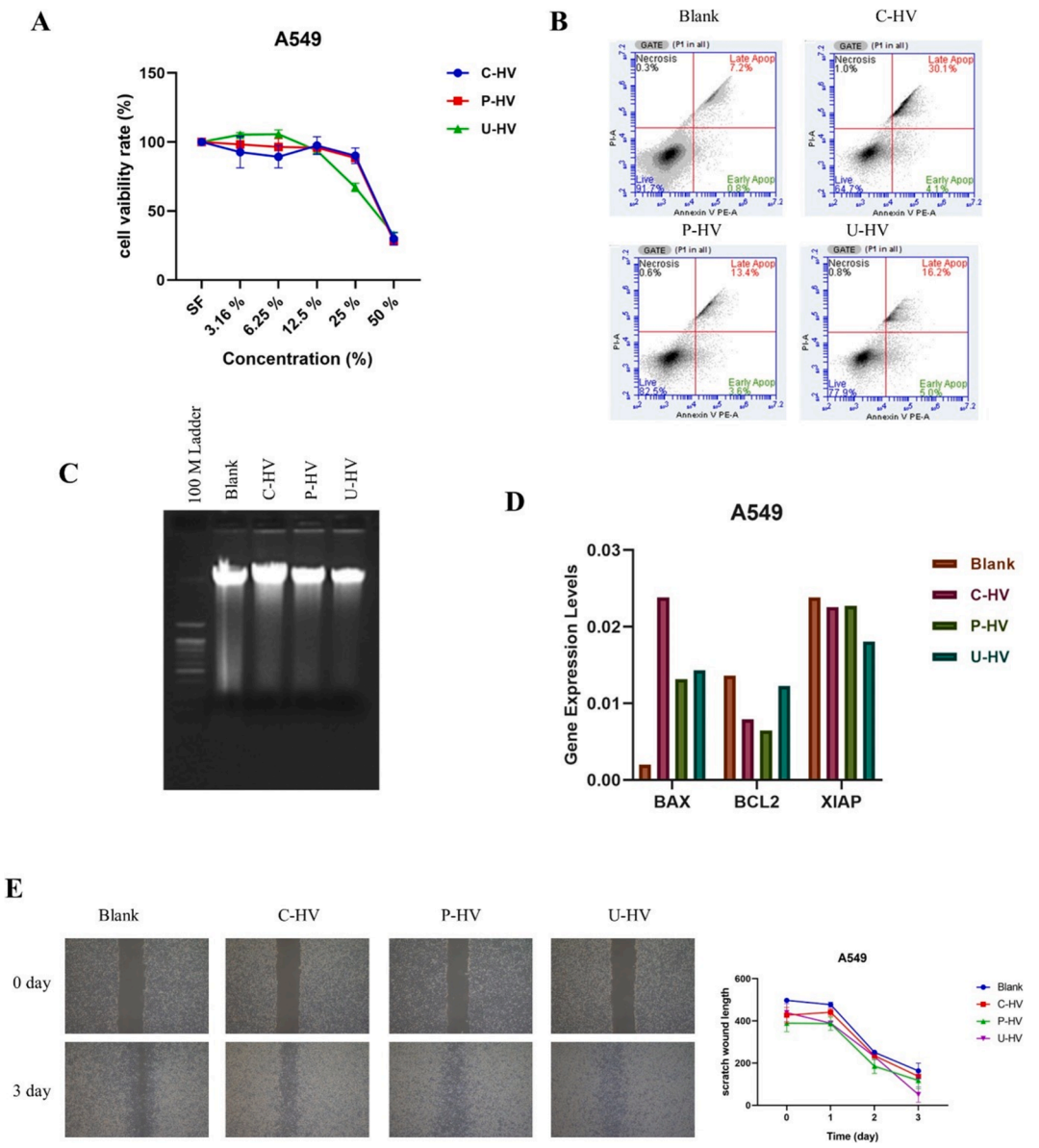

Fig. 4. Demonstration of the anticancer properties of C-HV, P-HV, and U-HV vinegar samples in A549 lung cancer cells. (A) MTT results for C-HV, P-HV, and U-HV vinegar samples. The effects of vinegar samples on cell death were shown using flow cytometry (B) and DNA fragmentation analysis (C). (D) Expression levels of apoptosis-related genes. (E) Wound healing assay showing effects of C-HV, P-HV, and U-HV samples on cell migration.

genes were analyzed. It was thus established that the C-HV, P-HV and U-HV vinegar samples exhibited efficacy at a minimum concentration of 25 % in A549 lung cancer cells (Fig. 4A). Later experiments were performed using the lowest concentration accordingly. The flow cytometry results determined that C-HV induced apoptosis in nearly 35 % of A549 cells, P-HV in 17 %, and U-HV in 21 % of the cells (Fig. 1B). Moreover, no significant difference was observed between the control and vinegar-treated groups regarding DNA fragmentation results (Fig. 4C). This can be explained by the low population of cells undergoing necrosis. According to the gene expression results, the expression level of the pro-apoptotic *BAX* gene was found to be highest in the C-HV samples compared to the control group. The expression levels of the *BCL-2* and *XIAP* genes were low in all three vinegar-treated groups (Fig. 4D). In accordance with the preceding findings, the wound healing assay results demonstrated that the group treated with C-HV samples exhibited a more substantial wound area than the control group (Fig. 4E).

It was found that C-HV, P-HV, and U-HV vinegar samples affected MCF-7 breast cancer cells at a concentration of 50 % (Fig. 5A). Furthermore, it was determined that the population of apoptotic cells in the vinegar-treated groups was approximately 30 % compared to the control group. The highest apoptotic rate was observed in the C-HV group (Fig. 5B). According to the gene expression results, the expression level of the *BAX* gene was highest in the C-HV group. In contrast, the expression levels of the BCL-2 and XIAP genes were lower in all three groups than in the control group (Fig. 5D). The low expression levels of the anti-apoptotic genes varied between the groups. However, no significant difference was observed in the DNA fragmentation results (Fig. 5C). The wound healing assay results provide further corroboration of the findings above, demonstrating that the wound in the C-HV group remained open even at the conclusion of the seven-day observation period, in contrast to the control group. This was followed by the P-HV and U-HV groups (Fig. 5E). According to the results from the colon cancer HT-29 cells, the effective dose of C-HV, P-HV, and U-HV vinegar samples was 50 % (Fig. 6A). The apoptosis results differed from the other two cell lines. Compared to the control group, higher levels of necrotic cell death were observed in the groups treated with C-HV, P-

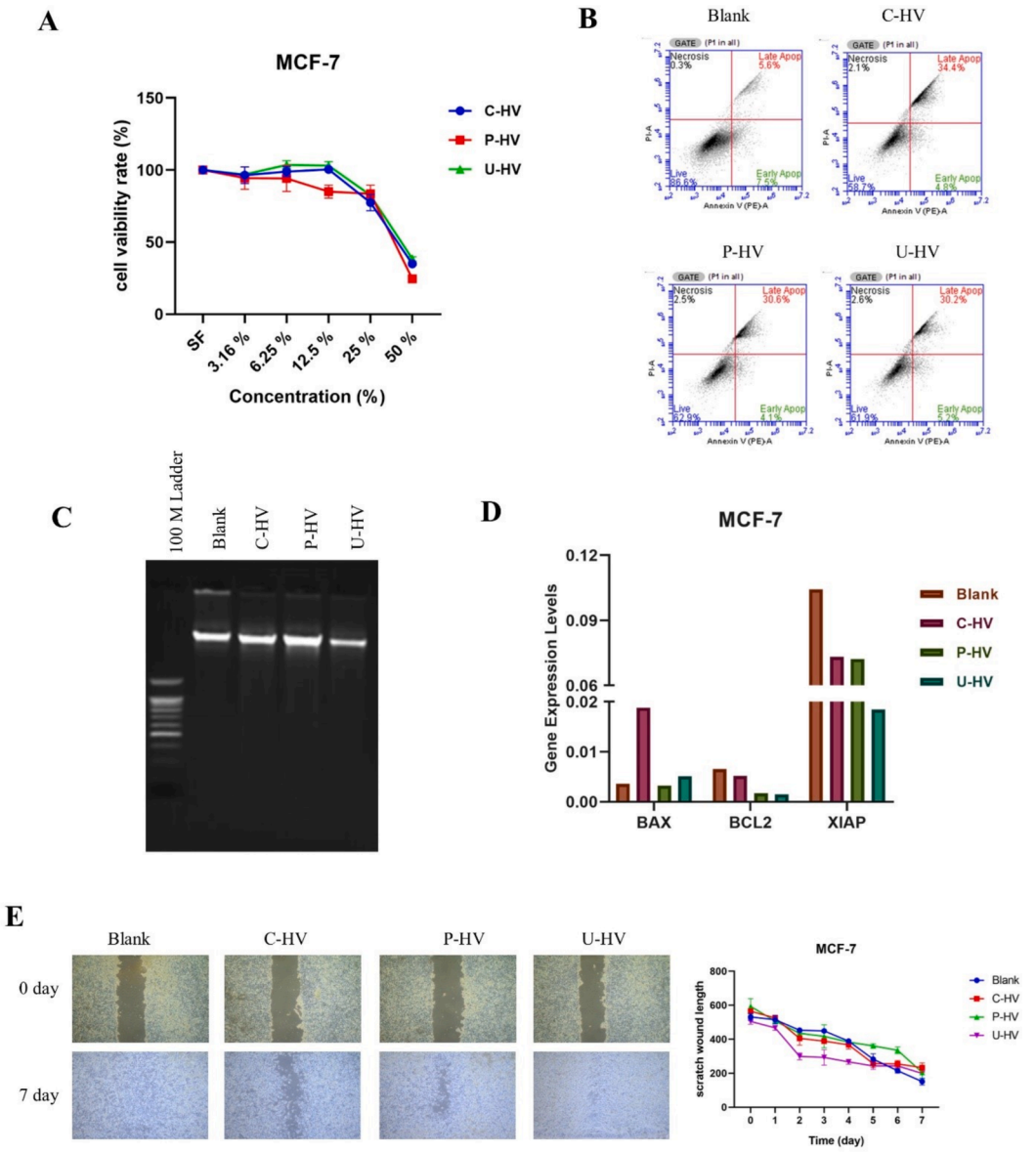

Fig. 5. Demonstration of the anticancer properties of C-HV, P-HV, and U-HV vinegar samples in MCF7 breast cancer cells. (A) MTT results for C-HV, P-HV, and U-HV vinegar samples. The effects of vinegar samples on cell death were shown using flow cytometry (B) and DNA fragmentation analysis (C). (D) Expression levels of apoptosis-related genes. (E) Wound healing assay showing effects of C-HV, P-HV, and U-HV samples on cell migration.

HV, and U-HV vinegar samples (Fig. 6B). The laddering pattern observed in the DNA fragmentation findings also supports this result (Fig. 6C). The wound healing assay results determined that the C-HV vinegar sample suppressed cell migration compared to the other groups (Fig. 6E). However, as with different cell types, it can be suggested that the suppression of cell migration in cancer cells is due to undesirable necrotic cell death that leads to inflammation. Comprehensive molecular studies may be required to investigate this further.

In this study, the cytotoxic effects of three vinegar samples obtained through different processing methods (C-HV, P-HV, and U-HV) on A549 (lung), MCF-7 (breast), and HT-29 (colon) cancer cell lines were investigated. Various assays evaluated cellular responses, including MTT, flow cytometry, DNA fragmentation, wound healing, and Real-Time PCR. Significant data were obtained regarding the therapeutic potential of these vinegar samples, which demonstrated potential anticancer effects by inducing apoptosis or inhibiting cell migration in cancer cells. This study explored the anticancer effects of hawthorn vinegar (C-HV, P-HV, and U-HV) on A549, MCF-7, and HT-29 cancer cell lines [60]. Experiments on the A549 cell line revealed that the C-HV vinegar sample exhibited a higher apoptotic effect at a 25 % concentration than the other (Fig. 4B). Flow cytometry analyses showed that C-HV induced approximately 35 % apoptosis, whereas this rate was 17 % for P-HV and 21 % for U-HV. This finding suggests that the apoptosis-inducing properties of C-HV are more potent than those of the other vinegar samples [61]. Gene expression analyses were performed to evaluate the molecular mechanism of apoptosis. It was observed that the expression level of the pro-apoptotic gene BAX significantly increased in the group treated with C-HV. In contrast, the expression levels of the anti-apoptotic genes BCL-2 and XIAP decreased (Fig. 4D). The high expression of BAX and the low levels of BCL-2 and XIAP explain the molecular basis of C-HV's pro-apoptotic effect [62]. However, the lack of a significant difference in DNA fragmentation tests (Fig. 4C) suggests that mechanisms other than apoptosis may also be active. This finding highlights the need for a deeper investigation into the balance between apoptosis and necrosis [63].

Our study's experiments on MCF-7 cells determined that vinegar samples were more effective at a 50 % concentration. Similarly, the C-HV vinegar sample exhibited the highest apoptotic effect, with apoptotic

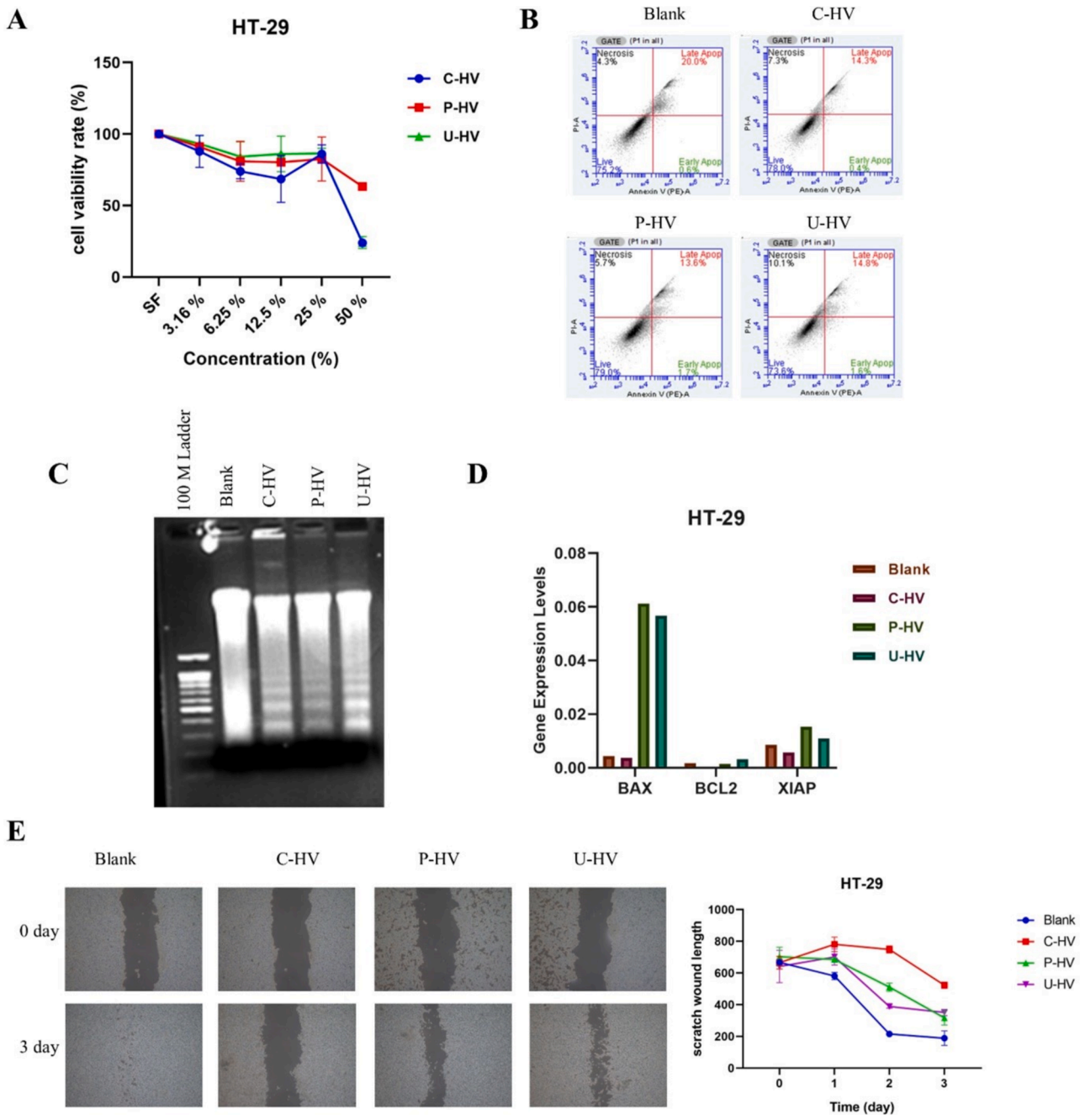

Fig. 6. Demonstration of the anticancer properties of C-HV, P-HV, and U-HV vinegar samples in HT-29 colon cancer cells. (A) MTT results for C-HV, P-HV, and U-HV vinegar samples. The effects of vinegar samples on cell death were shown using flow cytometry (B) and DNA fragmentation analysis (C). (D) Expression levels of apoptosis-related genes. (E) Wound healing assay showing effects of C-HV, P-HV, and U-HV samples on cell migration.

cells constituting approximately 30 % of the population. This finding is supported by the fact that the expression level of the pro-apoptotic gene **BAX** was the highest in the C-HV group compared to the other groups. Additionally, the low expression levels of the anti-apoptotic genes **BCL-2** and **XIAP** confirm the apoptosis-inducing potential of C-HV. In a study conducted by Nanda K. et al., the highest apoptosis rates were observed in the C-HV group, demonstrating that this vinegar sample has a stronger anticancer effect than the others. Moreover, wound healing assays supported these findings; cells treated with C-HV showed less wound closure, indicating suppressed cell migration. This suggests that C-HV can potentially limit the invasive capacity of tumor cells [64]. However, the absence of significant changes in DNA fragmentation analysis may indicate that the apoptotic mechanism in these cells was not fully activated or was suppressed through an alternative cell death pathway. These results are consistent with other studies suggesting that vinegar components may have metastasis-suppressing effects on breast cancer cells [65]. The results of the DNA fragmentation test demonstrated a pattern consistent with necrosis. This suggests that the vinegar samples' chemical compositions may significantly impact cell death mechanisms and that these mechanisms might vary across different cell types [66]. It is also hypothesized that the suppression of cell migration by C-HV could be associated with necrosis-induced inflammation. These observations indicate that vinegar samples may exhibit cell type-specific effects in different cancer types and, in some cases, may trigger undesirable necrotic cell death [67,68].

The study's overall findings reveal that while C-HV exhibits pro-apoptotic effects in A549 and MCF-7 cell lines, it induces higher rates of necrosis in HT-29 cells. This highlights the importance of the chemical composition of vinegar samples in influencing cell type-specific effects and the potential transition between apoptosis and necrosis [69]. The increased expression levels of the pro-apoptotic BAX gene and the decreased expression levels of BCL-2 and XIAP genes provide molecular support for the apoptotic induction of C-HV. However, for cell lines where necrosis predominates, more comprehensive molecular studies are required. Specifically, a detailed analysis of cytokine levels and inflammatory responses would be valuable to elucidate the mechanism of necrosis observed in HT-29 cells.

In conclusion, this study's findings demonstrate that the C-HV, P-HV, and U-HV vinegar samples have cancer cell type-specific anticancer effects. The effects of these vinegar samples on different cancer cell lines should be investigated more comprehensively at the molecular level. Understanding the transition between necrosis and apoptosis, in particular, will provide critical insights into the potential therapeutic applications of these products. Future studies, supported by in vivo models and clinical research, will further clarify the usability of these vinegar samples in cancer treatment.

4. Conclusion

This study investigated the effects of different processing methods of hawthorn vinegar on bioactive components and functional properties. It was shown that ultrasound application significantly increased bioactive parameters such as TPC, TFC, AA, DPPH radical scavenging activity, and CUPRAC reduction capacity. It was a more effective method than thermal pasteurization. In cell culture experiments, ultrasound-treated vinegar was found to have anticancer properties and support the apoptosis mechanism. In particular, ultrasound application increased the expression of pro-apoptotic genes and suppressed anti-apoptotic genes. It also showed beneficial effects by inhibiting migration in cancer cells. These findings indicate that ultrasound technology can effectively preserve and increase bioactive components in traditional foods and increase the potential of these products as functional foods. For future research, it is evaluated that ultrasound-assisted extraction offers significant opportunities in areas such as the discovery of new bioactive compounds for cancer and diabetes treatment, their clinical applications, and the elaboration of their mechanisms.

Ethical approval

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Selim Öğüt: Writing – original draft, Software, Methodology, Conceptualization. **Melikenur Türkol:** Writing – original draft, Methodology, Investigation, Data curation. **Seydi Yıkmış:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Esra Bozgeyik:** Writing – review & editing, Writing – original draft, Validation, Software. **Gholamreza Abdi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Emine Kocyigit:** Writing – review & editing, Writing – original draft, Validation, Software. **Rana Muhammad Aadil:** Writing – review & editing, Methodology, Investigation. **Nilay Seyidoglu:** Writing – review & editing, Writing – original draft. **Deniz Karakçı:** Writing – review & editing, Writing – original draft, Methodology. **Nazlı Tokatlı:** Writing – review & editing, Writing – original draft, Resources, Methodology, Data curation.

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.

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