Received: 30 May 2016 | Revised: 7 November 2016 | Accepted: 16 November 2016

DOI: 10.1002/bmc.3901

WILEY Biomedical Chromatography

RESEARCH ARTICLE

Gram (−) microorganisms DNA polymerase inhibition, antibacterial and chemical properties of fruit and leaf extracts of *Sorbus acuparia* and *Sorbus caucasica var. yaltirikii*

Halbay Turumtay[1] | Ahmet Midilli[2] | Emine Akyuz Turumtay[3] | Adem Demir[3] | Emine Kılıçkaya Selvi[3] | Emine Esra Budak[2] | Havva Er[3] | Fatih Kocaimamoglu[3] | Hüseyin Baykal[4] | Ali Osman Belduz[5] | Vagif Atamov[2] | Cemal Sandallı[2]

[1] Department of Energy System Engineering, Karadeniz Technical University, Trabzon, Turkey

[2] Department of Biology, Recep Tayyip Erdogan University, Rize, Turkey

[3] Department of Chemistry, Recep Tayyip Erdogan University, Rize, Turkey

[4] Department of Medicinal & Aromatic Plants, Recep Tayyip Erdogan University, Rize, Turkey

[5] Department of Biology, Karadeniz Technical University, Trabzon, Turkey

Correspondence
Cemal Sandalli, Department of Biology, Recep Tayyip Erdogan University, 53100 Rize, Turkey.
Email: csandalli@yahoo.com

Funding information
The Scientific and Technical Research Council of Turkey, Grant/Award Number: TUBİTAK-113Z054

Abstract

Investigation of novel plant-based agents might provide alternative antibiotics and thus fight antibiotic resistance. Here, we measured the ability of fruit and leaf extracts of *Sorbus aucuparia* (*Sauc*) and endemic *Sorbus caucasica* var. *yaltirikii* (*Scau*) to inhibit nonreplicative (Klenow Fragment-KF and Bacillus Large Fragment-BLF) and replicative (DnaE and PolC) bacterial DNA polymerases along with their antimicrobial, DPPH free radical scavenging activity (RSA), and chemical contents by total phenolic content and HPLC-DAD analysis. We found that leaf extracts had nearly 10-fold higher RSA and 5-fold greater TPC than the corresponding fruit extracts. All extracts had large amounts of chlorogenic acid (CGA) and rutin, while fruit extracts had large amounts of quercetin. Hydrolysis of fruit extracts revealed mainly caffeic acid from CGA (caffeoylquinic acid) and quercetin from rutin (quercetin-3-*O*-rutinoside), as well as CGA and derivatives of CGA and *p*-coumaric acid. Plant extracts of *Sorbus* species showed antimicrobial activity against Gram-negative microorganisms. *Scau* leaf extracts exhibited strong inhibition of KF activity. *Sauc* and *Scau* leaf extracts also strongly inhibited two replicative DNA polymerases. Thus, these species can be considered a potential source of novel antimicrobial agents specific for Gram-negative bacteria.

KEYWORDS

inhibition, nonreplicative DNA polymerase (KF and BLF), organic solvents, primer extension assay, replicative DNA polymerase (DnaE and PolC), *Sorbus*

1 | INTRODUCTION

Although antibiotics have protected millions of lives since their discovery, antibiotic-resistant bacteria represent a growing challenge (Coates, Hu, Bax, & Page, 2002). Bacteria possess certain mechanisms to overcome antibiotic pressure, which then facilitates the selection of resistant organisms (Alekshun & Levy, 2007; Walsh, 2000; Wright, 2003). The development of antibiotic resistance is the inevitable outcome of antibiotic utilization, which limits the efficacy and useful life of antibiotics. Correct utilization of antibiotics will slow down the development of bacterial resistance, but it cannot prevent it totally. Ongoing discovery and development of new antibiotics will guarantee therapy for bacterial infections now and in the future (Chopra, Hodgson, Metcalf, & Poste, 1997; Projan, 2002). The antibiotics currently in use affect a limited number of mechanisms within the bacterial cell; thus, antibiotics targeting novel cellular mechanisms of antibiotic resistance are urgently needed (Habich & von Nussbaum, 2006).

Based on our recent review of the literature, antibiotics that target bacterial replication enzymes have not yet been reported. DNA replication, which occurs via DNA polymerases, is an essential process that occurs prior to bacterial cell division during pathogen proliferation. DNA polymerases (DNA-dependent DNA polymerase, E.C. 2777)

Abbreviations used: BLF, *B. subtilis* large fragment; CA, caffeic acid; CCGA, cryptochlorogenic acid; CGA, chlorogenic acid; CGAE, chlorogenic acid equivalent; DE, diethyl ether; DMSO, dimethylsulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Ds, derivatives; EA, ethyl acetate; KF, Klenow fragment; NCGA, neochlorogenic acids; *p*-CA, *p*-coumaric acid; QE, quercetin equivalent; RSA, free radical scavenging activity; *Sauc*, *Sorbus aucuparia*; *Scau*, *Sorbus caucasica* var. *yaltirikii*; SC_{50}, value of the concentration of sample required to scavenge 50% of DPPH radicals (mg sample per mL methanol); TPC, total phenolic content.

catalyze the addition of deoxyribonucleotides to the 3′-hydroxyl terminus of primed, double-stranded DNA (Kornberg & Baker, 1992). All living organisms possess multiple DNA polymerases, which are classified into six families (A, B, C, D, X and Y). DNA polymerases that share sequence homology with *Escherichia coli* DNA polymerase I, II and III have been assigned to the A, B, and C families, respectively (Delarue, Poch, Tordo, Moras, & Argos, 1990). While DNA polymerase I is a nonreplicative enzyme, DNA polymerase III is the primary replicative enzyme in bacterial cells. All known DNA polymerases from the same family share significant sequence homology and they are structurally and functionally similar to each other. However, there are some differences among the families in their biochemical properties such as polymerization rate, dideoxyribonucleotide triphosphate sensitivity, strand displacement synthesis, RNA-dependent DNA synthesis, DNA repair and inhibition (Kenneth, Arnold, & Cameron, 2008; Rothwell & Waksman, 2005; Wu, Beard, Pedersen, & Wilson, 2014).

Besides their primary metabolites such as proteins, carbohydrates, lipids and nucleic acids, plants also produce secondary metabolites, which have important functions both in plants and in humans. Secondary metabolites of many plant tissues have been used as new antibacterial agents. Identification of plant-based agents that exhibit antimicrobial activity may help to identify new antibiotics, which in turn, is essential in the fight against antibiotic resistance. Plant extracts are typically prepared in organic solvents; in order to determine the effects of the plant extracts it is critical to rule out the effect of organic solvents on the cellular function under investigation. In the current study, we first investigated the inhibitory effects of six different organic solvents ranging from highly polar to nonpolar [methanol, butanol, ethyl acetate, diethyl ether, hexane and dimethylsulfoxide (DMSO)] against replicative and nonreplicative DNA polymerases. Unlike the other solvents, DMSO dissolves both polar and nonpolar compounds and is used in polymerase chain reaction at optimized concentrations because of its ability to inhibit DNA secondary structure formation in template and primer at high concentrations (Chakrabarti & Schutt, 2001; Ralser et al., 2006). Next, we determined the ability of leaf and fruit extracts of two *Sorbus* species, *Sorbus aucuparia* L. (*Sauc*) and *Sorbus caucasica* Z. var. *yaltirikii* G. (*Scau*), to inhibit DNA polymerase I and III from *E. coli* and *B. subtilis*. The *Sorbus* genus from the Rosaceae family has more than 250 species, many of which have been widely used in traditional medicine for the treatment of chronic tracheitis, tuberculosis and edema (Aldasoro, Aedo, Navarro, & Garmendia, 1998). *Sorbus aucuparia* L. (rowanberry) is a common plant producing a yellowish, wild berry. Its origins are Siberia and northern Europe and it also commonly found in northeast Anatolia (Devis, 2008; Gil-Izquierdo & Mellenthin, 2001). *Sorbus caucasica* Z. var. *yaltirikii* G. is the only rowan species native to Turkey. Rize-Pazar and Artvin-Yusufeli are known places where it grows (Goksin, 1977).

2 | MATERIALS AND METHODS

2.1 | Collection of plant samples and extraction preparation

The leaves and fruits of *Sauc* and *Scau* were collected in Alpe Gera (Başyayla, Rize, Turkey) during the summer of 2014; samples were stored at −70°C. A small amount of each sample (0.5 g) was used to prepare extract. First, samples were pulverized under liquid nitrogen using a mortar and pestle. The sample powder was then extracted with 6 mL of methanol at 20°C for 2 h in an ultrasonic bath (Elma Clean Box, Elma) and then centrifuged at 4000 rpm for 20 min at 4°C. Supernatants were transferred into a new Falcon tube and evaporated (EZ-2 Evaporator, GeneVac). Dried extracts were dissolved in 0.5 mL of methanol to achieve a ratio of extract from 1 g fresh material/1 mL methanol. Next, 1 µL of each extract was used in primer extension assays (using 1 µL of extract ensured 10% of each solvent in final concentration in reaction) to investigate their effects on replicative and nonreplicative DNA polymerases. Leaf extracts were referred to as *Sauc*-L and *Scau*-L, while fruit extracts were named *Sauc*-F and *Scau*-F. The same extraction was also performed with DMSO and only used for antimicrobial assays. Methanol extracts were used for primer extension, the total phenolic compounds and radical scavenging activity assays.

2.2 | Preparation of DNA polymerases

The Klenow fragment (KF, *E. coli* DNA polymerase I) and large fragment (BLF, *B. subtilis* DNA polymerase I) were purchased from ThermoScientific (EP0422) and New England Biolabs (M0275S), respectively. DnaE and PolC were cloned from the genome of *E. coli* K12 and *B. subtilis* ATCC66333, respectively. Briefly, bacteria were grown in Luria–Bartani medium at 37°C; genomic DNA was purified using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Both DnaE and PolC forward primers were designed with a CACC sequence to allow in-frame ligation into the pET100/D-TOPO expression vector (Invitrogen; Table 1). Both genes were amplified using Expand High Fidelity *Taq* DNA polymerase (Fermentas), directly ligated into the TOPO-100 expression vector, and transformed into *E. coli* One Shot® TOP10. Recombinant plasmids harboring the polymerase genes were screened, purified and separately transformed into *E. coli* One Shot® BL21 Star™ (DE3) for polymerase overexpression. The expression of DnaE and polC polymerases was carried out in *E. coli* One Shot® BL21 Star™ (DE3) cells as described by Sandalli, Canakci, Demir, Modak, and Belduz (2010). Protein concentrations were determined by the NanoDrop (ND-1000 Spectrophotometer) method.

TABLE 1 Antimicrobial activity of *Sorbus* DMSO extracts

		Gram (−)			Gram (+)		
Extracts	(mg/mL)	*E. coli*	S. typ	P. aer	S. aur	E. fec	B. sub
*Sauc*L	12	6	6	8	—	—	—
*Scau*L	12	—	—	10	—	—	—
*Sauc*F	30	6	—	8	—	—	—
*Scau*F	30	6	6	10	—	—	—
Amp	10	10	10	18	10	35	15

E. coli, *Escherichia coli* ATCC25922; *S. typ*, *Salmonella typhimurium* ATTC14028, *P. aur*, *Pseudomonas aeruginosa* ATCC 27853; *S. aur*, *Staphylococcus aureus* ATCC25923; *E. fec*, *Enterococcus faecalis* ATTC29212; *B. sub*, *Bacillus subtilis* ATCC6633; *Sauc*, *Sorbus aucuparia*; *Scau*, *Sorbus caucasica* var. *yaltirikii*; Amp, ampicillin; L, leaf; F, flower; —, no activity.

2.3 | Primer extension assays

DNA polymerase activity in the presence and absence of plants extracts was investigated by primer extension assay. The reactions were performed in 10 µL volume with the appropriate polymerase (0.5 units for nonreplicative polymerases and 25 nM for replicative DNA polymerases), 1× polymerase activity buffers, $MgCl_2$ (1.5 mM for nonreplicative polymerases, 10 mM for replicative polymerases), 100 ng 45/20-mer DNA (5′- end labeled by Cy5, Table 1), and 250 µM each dNTP; reactions were carried out for 10 min at 37°C for KF, 65°C for BLF, and 30°C for DnaE and polC. The reactions were terminated by addition of 10 µL of 2× stop solution [20 mM EDTA, 0.2% (*w*/*v*) SDS, 80% (*v*/*v*) formamide and 0.008% (*w*/*v*) each of bromophenol blue and xylene cyanol] and heated at 95°C for 5 min. Six microliters of reaction mixture was loaded onto a 16% polyacrylamide–8 M urea gel and the products were separated at 1500 V for 3 h. The resulting gel was visualized by Typhoon FLA9500 biomolecular imager (Amersham Biosciences).

We have also investigated the inhibitory effects of six organic solvents (methanol, butanol, hexane, ethyl acetate, DMSO and diethyl ether) at the different concentrations (5, 10, 20 and 40%) on both DNA polymerases to control for solvent effects on polymerase activity.

2.4 | Antimicrobial activity

We evaluated the antimicrobial effects of each extract on Gram-negative (*E. coli* ATCC25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATTC14028) and Gram-postive (*Staphylococcus aureus* ATCC25923, *B. subtilis* ATCC6633, *Enterococcus faecalis* ATTC29212) bacteria. All plant extracts were prepared in DMSO at 12 mg/mL (leaves) and 30 mg/mL concentration (fruits); assays were performed according to Ahmad et al. (1998) using the agar-well diffusion method. Briefly, each bacterial culture was suspended in Meuller-Hinton Broth (Difco, Detroit, MI, USA) and diluted to 10^{-6} colony forming units (cfu) per mL. They were 'flood-inoculated' onto the surface of Meuller–Hinton agar plates and then dried. Wells (5 mm diameter) were cut from the agar using a sterile cork-borer, and 50 µL of the extract were delivered into the wells. The plates were incubated for 18 h at 35°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Ampicillin (Sigma) (10 µg/mL) was used as standard drug and DMSO was used as a solvent control. Results were determined by the size of the inhibition zone.

2.5 | Determination of total phenolic content

The total phenolic content (TPC) of the methanol extracts was measured using Folin–Ciocalteu's phenol reagent. Chlorogenic acid and quercetin were chosen to represent phenolic standards to generate a standard curve in a range from 0.019 and 0.600 mg/mL ($r^2 = 0.998$) (Singleton, 1985). Only crude methanol extracts were analyzed for TPC. The extracts were diluted 1:20 with methanol to ensure that the TPC would fall within the dynamic range of calibration curves. Briefly, 20 µL of plant extract, 400 µL of 0.5 M Folin–Ciocalteu reagent, and 680 µL of distilled water were vigorously mixed. This mixture was incubated for 3 min at room temperature before the addition of 400 µL of Na_2CO_3 (10%). The absorbance of the mixture was measured at 760 nm with a UV–vis detector (Labomed Inc., Culver City, USA) after 2 h. The concentration of total phenolic compounds was calculated as mg of chlorogenic acid equivalent (CGAE) and mg of quercetin equivalent (QE) respectively for phenolics and flavonoids per gram of dried extracts.

2.6 | Free-radical scavenging activity assay (DPPH)

The free-radical scavenging activity of methanol extracts against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was spectrophometrically determined at 517 nm (Molyneux, 2004). Briefly, 0.30 mL plant extract was mixed with 0.30 mL 0.1 mM DPPH in methanol. Gallic acid, chlorogenic acid (CGA), and quercetin were used as standards. Results are reported as SC_{50} values, indicating the concentration of sample required to scavenge 50% of DPPH radicals (mg sample per mL methanol).

2.7 | Identification of phenolic compounds in plant extracts using HPLC-DAD

The extraction procedure was optimized for HPLC analysis. Fresh samples were pulverized with a commercial blender (Waring, Osaka, Japan) and 10 g from fruit and leaf tissues of *Sauc* and *Scau* were extracted with 100 mL of methanol for 2 h in the shaker at 28°C. Extracts were centrifuged at 4000 rpm for 20 min at 4°C. Supernatants were transferred into a new falcon tube and 10 mL of the extracts were evaporated (EZ-2 Evaporator, GeneVac) until dry. Dried extracts were dissolved in 2 mL of methanol and labeled *Sauc*F-M, *Scau*F-M, *Sauc*L-M and *Scau*L-M (F and L denote fruit and leaf, respectively). The rest of the methanol extracts were evaporated until dry and dissolved in 100 mL of water prior to liquid–liquid extraction. A 10 mL aliquot of hexane was used twice for defatting; 2 × 10 mL of diethyl ether (DE) and 2 × 10 mL of ethyl acetate twice (EA) were used to fractionate the extracts. Organic fractions (DE and EA) were combined in the same flask, evaporated, and dissolved with 1 mL of methanol and labeled *Sauc*F-O, *Scau*F-O, *Sauc*L-O and *Scau*L-O. The water fractions were evaporated until dryness and dissolved with 5 mL methanol and named as *Sauc*F-W, *Scau*F-W, *Sauc*L-W and *Scau*L-W. Acidic hydrolysis was also applied to the fruit extracts. The methanol extracts of the fruit were dissolved with 5 mL methanol and 5 mL of 4 M hydrochloric acid and boiled at 100°C for 2 h. Hydrolyzed extracts were cooled and extracted with 2 × 10 mL of DE, and 2 × 10 mL of EA. Organic fractions were combined in the same flask, evaporated, dissolved with 2 mL of methanol and labeled *Sauc*F-HO and *Scau*F-HO. The water fractions were evaporated until dry, dissolved with 2 mL of methanol and named *Sauc*F-HW and *Scau*F-HW. Extracts were diluted with water by adjusting to 56% methanol in water prior to HPLC-DAD analysis.

The chromatographic analyses were performed using a Dionex (Thermo Scientific, Germering, Germany) Ultimate 3000 HPLC system equipped with an Ultimate 3000 Degasser, an Ultimate LPG-3400SD Pump, an Ultimate WPS-3000TSL autosampler, an Ultimate 3000 DAD and an Ultimate TCC-3000SD column compartment. An Agilent reverse-phase C_{18} column (PLRP-S 150 × 4.6 mm i.d., 5 µm particle diameter 100 Å) was used. Gradient elution separation was performed with mobile phase A (2% acetic acid in water) and B (70% acetonitrile)

using the following time-set; 0–3 min 5.5% B; 3–10 min 5.5–23% B; 10–20 min 23–31% B; 20–30 min 31–56% B; 30–40 min 56–85% B; 40–45 min 85–5.5% B; 45–60 min 5.5% B. The injection volume was 10 µL, the column temperature was 30°C, and the flow rate was 1.0 mL/min. The DAD detector recorded spectra between 200 and400 nm. Chromatograms were recorded at 254 nm for protocatechuic acid, *p*-hydroxy benzoic acid, vanillic acid, rutin and ellagic acid; at 280 nm for gallic acid, syringic acid, paeonol, and thymol; at 315 nm for chlorogenic acid (3-caffeoylquinic acid), caffeic acid, *p*-coumaric acid and ferulic acid; and at 370 nm for apigenin, myricetin, fisetin, quercetin, kaempferol and isorhamnetin. Gallic acid, protocatechuic acid, CGA, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, syringic acid and thymol were prepared at 1000 µg/mL in 50:50 methanol–water. Ellagic acid, rutin, quercetin, apigenin and kaempferol were prepared at 1000 µg/mL in methanol. Myricetin and fisetin were prepared at 2000 µg/mL and isorhamnetin at 250 µg/mL in methanol. Paeonol was prepared at 833 µg/mL in 1 mL methanol with 200 µL of 1 M NaOH. Calibration curves were calculated using standard mixtures at 0.125, 0.5, 1, 2, 5, 10, 20, 30 and 40 mg/L in 56% methanol. This method was validated for repeatability by injecting 0.5 µg/mL of the standard mixture 7 times (2 µg/mL for rutin, quercetin and isorhamnetin) and percentage relative standard deviation (RSD) of peak areas and retention times was calculated. The limit of detection (LOD) and quantification (LOQ) were calculated using the ratio of standard deviation of the

TABLE 2 Phenolic compounds in the nonhydrolyzed and hydrolyzed fruit extracts of two *Sorbus* species by HPLC-DAD

Compounds			mg Standard/g extract									
			SaucF			*ScauF*			*Sauc*F-H		*Scau*F-H	
No.	Retention time	Name	M	O	W	M	O	W	O	W	O	W
1	7.6	CGA -D1	1.9	1.3	1.5	3.3	1.8	2.9	208.7	n.d.	n.d.	n.d.
2	9.4	CGA-D2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	59.8	n.d.	46.6
3	9.7	CGA-D3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	37.9	n.d.	30.5
4	10.7	CGA-D4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	367.7	17.2	n.d.	9.5
5	11.1	CGA	6.7	9.4	5.7	5.5	4.3	5.4	1279.9	66.7	490.4	63.1
6	11.6	CGA-D5	n.d.	n.d.	n.d.	0.9	0.9	0.8	n.d.	n.d.	88. 9	n.d.
7	11.8	*p*-CA-D1	0.1	1.7	0.1	n.d.	0.1	n.d.	n.d.	n.d.	95.2	n.d.
8	12.1	CA-D1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.2	n.d.	46.9
9	12.6	CA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3550.7	42.6	690.6	37.3
10	13.1	*p*-CA-D2	0.1	n.d.	0.1	0.1	0.2	0.1	n.d.	n.d.	n.d.	n.d.
11	13.5	Rutin-D2	0.5	0.9	0.5	n.d.	n.d.	0.2	n.d.	n.d.	n.d.	n.d.
12	13.7	CGA-D6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	17.6	n.d.	26.1
13	14	Rutin-D3	0.6	0.7	0.5	0.7	0.5	0.8	n.d.	n.d.	n.d.	n.d.
14	15.1	Rutin	0.2	0.3	0.2	1	0.8	1.4	n.d.	n.d.	n.d.	n.d.
15	15.1	CGA-D7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1559.8	80.1	n.d.	61.2
16	16	Rutin-D4	0.3	1.6	0.3	1	1.2	1	n.d.	n.d.	n.d.	n.d.
17	16.1	Rutin-D5	0.4	2.1	0.3	n.d.	0.8	0.6	n.d.	n.d.	n.d.	n.d.
18	16.5	CGA-D8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.8	n.d.	n.d.
19	16.7	Rutin-D6	n.d.	n.d.	n.d.	0.4	0.7	0.6	n.d.	n.d.	n.d.	n.d.
20	16.7	*p*-CA-D3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	349.2	n.d.	322.6	n.d.
21	17.6	Rutin-D7	0.5	3.8	0.4	n.d.	1.1	0.6	n.d.	n.d.	n.d.	n.d.
22	18	Rutin-D8	0.5	4	0.4	0.4	1	0.4	n.d.	n.d.	n.d.	n.d.
23	19.5	CA-D3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.1	n.d.	511.5	n.d.
24	20.7	CGA-D9	0.1	1.9	0.1	1.3	9.2	0.7	n.d.	n.d.	n.d.	n.d.
25	24.3	CGA-D11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13017.2	21.7	1769.2	n.d.
26	24.4	*p*-CA-D4	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	n.d.	n.d.	n.d.
27	24.6	*p*-CA-D5	n.d.	n.d.	n.d.	n.d.	0.3	n.d.	n.d.	n.d.	n.d.	n.d.
28	27.9	Rutin-D10	n.d.	n.d.	n.d.	n.d.	0.4	n.d.	n.d.	n.d.	n.d.	n.d.
29	28.1	Quercetin	n.d.	0.1	n.d.	n.d.	0.1	n.d.	222.2	n.d.	n.d.	n.d.
30	28.8	CGA-D12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	219.8	n.d.	n.d.
31	30.9	*p*-CA-D6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	772.2	n.d.	646.1	n.d.
Total rutin/rutin-Ds			3	13.4	2.6	3.5	6.5	5.6	0	0	0	0
Total CGA/CGA-Ds			8.7	12.6	7.3	11	16.2	9.8	16433.3	530.6	2259.6	237.0
Total			11.9	27.8	10.1	14.6	23.6	15.5	21337.7	584.4	4525.6	321.2

CGA, chlorogenic acid; *p*-CA, *p*-coumaric acid; CA, caffeic acid; Ds, derivatives; n.d., not determined; M, Methanol extract; O, Organic phase; W, Water phase.

area from this repeated analysis to the calibration slope values. The LOD and LOQ were calculated using 3 and 9 times the signal-to-noise ratio, respectively.

3 | RESULTS AND DISCUSSION

3.1 | Profile of phenolic compounds in *Sorbus* extracts as determined by HPLC-DAD

The external calibration method by HPLC-DAD was linear for the 19 standard phenolic compounds with coefficients of determination (R^2) at least 0.999. Repeatability of the retention time and areas were up to 95%, with the exception for gallic acid, which was located closest to the solvent peak. Quite small detection limits were obtained for all standards with the highest values for gallic acid and isorhamnetin, whose LOQ values were 0.30 and 0.40 μg/mL, respectively.

The extracts from the two *Sorbus* species were analyzed using this optimized HPLC-DAD method. The aim of HPLC-DAD analysis of the phenolic compounds in these plant extracts was the elucidation of the active compound(s) responsible for their inhibitory effects on KF, BLF, DnaE and PolC. All peaks in the chromatograms of extracts were either identified and quantified by this method or characterized and tentatively identified based on their elution orders and UV spectra. In addition, characterized peaks were quantified using the calibration equations of the chemically closest standards used in this method.

The results of the HPLC-DAD are displayed in Tables 2 and 3. The primary phenolic compounds found in the extracts were CGA and rutin; fruit extracts also had quercetin. The retention times of many other peaks did not match the current standards; therefore, the characterization of unknown peaks was performed using their UV spectra. These peaks were identified as the derivatives (Ds) of CGA, rutin, caffeic acid (CA) and *p*-coumaric acid (*p*-CA). Twelve CGA, 10 rutin, six *p*-CA and three CA Ds were characterized and quantified as the equivalent of the original compound. Acidic hydrolysis of the fruit extracts was performed to cleave sugar groups from the rutin derivatives and quinic acid groups from the CGA-Ds to confirm the characterization. Quercetin was greatly increased in the hydrolyzed extract of *Sauc* that is the aglycone of rutin, while rutin disappeared in the hydrolyzed extracts (Table 2). This is also an indication of sufficient hydrolysis. Furthermore CA, which is the main group of CGA (caffeoyl quinic acid), and two CA-Ds appeared after hydrolysis (Figure 1 and Table 2). Both the number and amount of CGA and its derivatives were greatly increased via hydrolysis (Figure 1 and Table 2). The interpretation of the identification and characterization of the phenolic compounds of these two species were compared with other literature reports and found to be quite consistent. Gil-Izquierdo and Mellenthin (2001) elucidated the flavonols and chlorogenic acid derivatives in *Sauc* juice. CGA and neochlorogenic acids (NCGA) were found to be the most abundant phenolic compounds, while quercetin-3-*O*-hexose-hexosides were the most abundant flavonols (Gil-Izquierdo & Mellenthin, 2001). Olszewska, Presler, and Michel (2012) identified some chlorogenic acid derivatives such as NCGA and cryptochlorogenic acid (CCGA) in the *Sauc* fruit extracts. In the current study, any of the chlorogenic acid derivatives characterized in the extracts could be considered NCGA and CCGA based on these reports. Phenolic compounds

TABLE 3 Phenolic compounds in the leaf extracts of two *Sorbus* species by HPLC-DAD

			mg Standard/g extract					
No.	Retention time	Compounds	*Sauc*L-M	*Sauc*L-O	*Sauc*L-W	*Scau*L-M	*Scau*L-O	*Scau*L-W
1	7.6	CGA-D1	0.8	0.4	0.9	0.8	0.5	1.2
2	11.1	CGA	9.6	7.0	15.4	3.2	3.3	5.2
3	11.6	CGA-D5	n.d.	n.d.	n.d.	0.4	0.3	0.4
4	13.0	Rutin-D1	n.d.	n.d.	n.d.	1.9	n.d.	2.6
5	13.1	*p*-CA-D2	0.4	n.d.	0.9	n.d.	n.d.	n.d.
6	13.2	CA-D2	n.d.	n.d.	n.d.	0.6	n.d.	0.8
7	13.5	Rutin-D2	14.2	10.1	21.1	1.0	n.d.	1.4
8	14.0	Rutin-D3	10.1	7.0	15.1	8.5	7.9	13.0
9	15.1	Rutin	11.6	9.6	17.4	23.5	21.1	36.4
10	16.0	Rutin-D4	9.9	15.2	14.0	8.0	9.7	12.3
11	16.1	Rutin-D5	15.7	21.3	22.4	9.0	10.9	12.7
12	16.7	Rutin-D6	1.9	n.d.	n.d.	6.1	5.7	9.4
13	17.6	Rutin-D7	9.7	11.9	n.d.	5.1	4.9	9.5
14	18.0	Rutin-D8	3.4	4.6	15.1	2.6	2.4	5.8
15	18.9	Rutin-D9	0.5	1.0	5.3	n.d.	n.d.	n.d.
16	20.7	CGA-D9	3.0	10.2	3.9	4.7	10.7	7.0
17	22.1	CGA-D10	n.d.	n.d.	n.d.	n.d.	0.6	0.4
18	24.4	*p*-CA-D4	n.d.	n.d.	n.d.	n.d.	0.4	0.1
19	24.6	*p*-CA-D5	n.d.	n.d.	n.d.	n.d.	0.5	0.2
Total rutin/rutin-Ds			76.9	80.6	110.5	65.7	62.5	103.1
Total CGA/CGA-Ds			13.4	17.7	20.2	9.0	15.3	14.1
Total			90.8	98.3	131.6	75.3	78.6	118.4

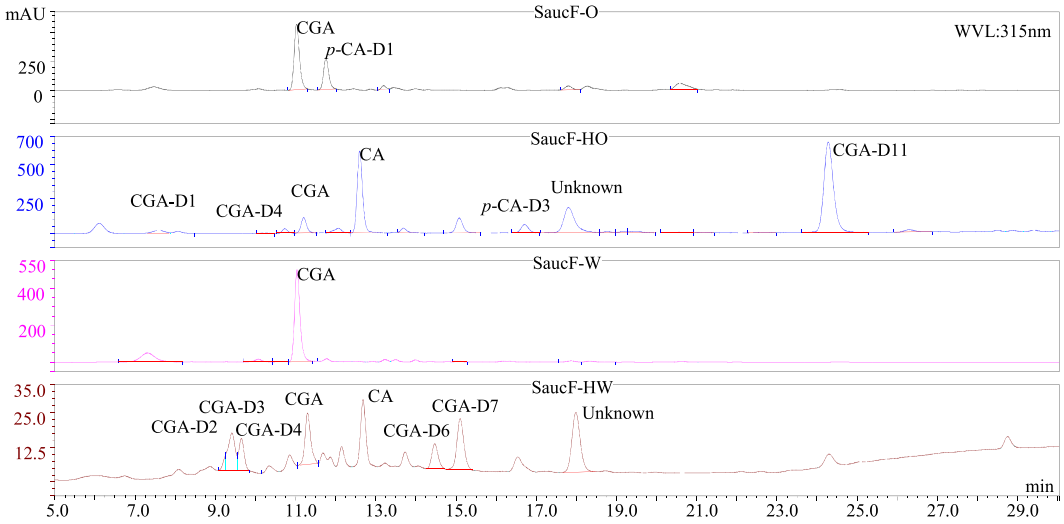

FIGURE 1 HPLC-DAD chromatograms of hydrolyzed extracts of *Sorbus aucuparia* (*Sauc*) fruits at 315 nm

of *Sauc* fruits were extracted using 50% methanol with 1.2 M hydrochloric acid and analyzed using HPLC-DAD by Hakkinen, Karenlampi, Heinonen, Mykkanen, and Torronen (1999). Ferulic acid (52.6%) was most prevalent followed by quercetin (26.9%), CA (13.4%), *p*-CA (4.6%) and ellagic acid (1.7%) among the phenolic acids and flavonoids aglycones studied. In contrast, although ferulic acid was included in our phenolic standards, it was not detected in the extracts. Phenolic profiles of *Sorbus* species including *Sauc* and *Scau* revealed their main compounds to be NCGA and followed by CGA, along with isoquercitrin, rutin and hyperoside according to HPLC-DAD analysis (Raudonis, Raudone, Gaivelyte, Viskelis, & Janulis, 2014). Similar results were obtained for leaf extracts, with the most prevalent compounds being CGA followed by NCGA along with the CGA derivatives and sugar-attached quercetin derivatives in the extracts as detected by UPLC-QTOF-MS (Raudone et al., 2015). The number of phenolic compounds was higher in the leaf extracts than the fruit extracts. Leaf extracts contained the most rutin derivatives while the fruit extracts had the most chlorogenic acid derivatives (Figure 2 and Table 3).

3.2 | Effect of *Sorbus* extracts on replicative and nonreplicative DNA polymerase activity

The structures of replicative DNA polymerase IIIs and nonreplicative DNA polymerase Is are strongly conserved in both Gram-positive and Gram-negative pathogens. These DNA polymerases have been considered targets for antibiotic development for a long time Wright and Brown (1999). Identification of new antibacterial agents in plant tissues that target bacterial DNA polymerase IIIs in Gram-positive and Gram-negative bacteria is important in for the development of new antibiotics.

We first investigated the inhibitory effects of six organic solvents (methanol, butanol, hexane, ethyl acetate, DMSO and diethyl ether) at several different concentrations (5, 10, 20 and 40%), which allowed us to control for the effect of the solvent used for extraction on DNA polymerase activity (data not shown). Although there was no polymerase activity at all concentrations of butanol, DE and hexane did not affect the activity of any polymerase at any concentration. DMSO did not affect the activity of any polymerase at a concentration of 10%, but

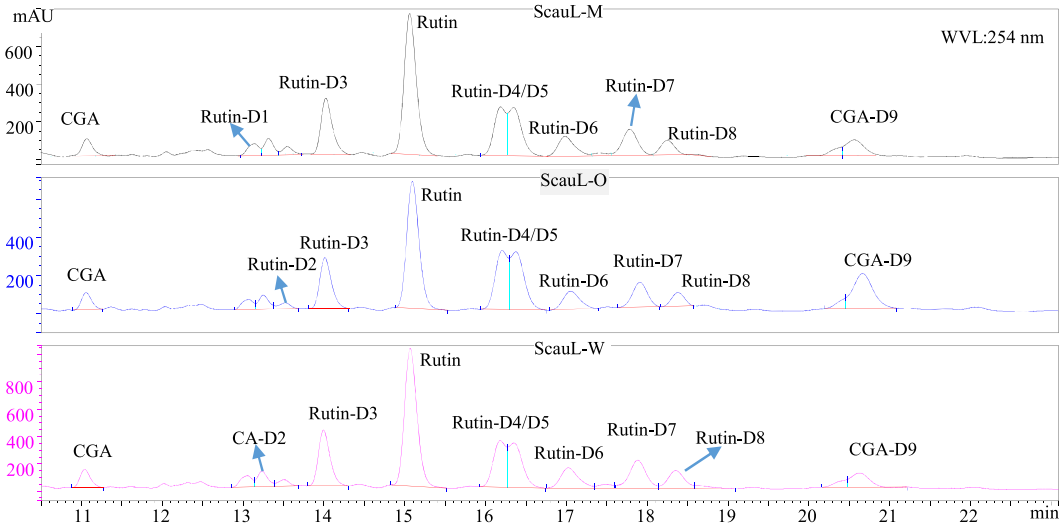

FIGURE 2 HPLC-DAD chromatograms of *Sorbus caucasica* var. *yaltirikii* (*Scau*) leaf extracts at 254 nm

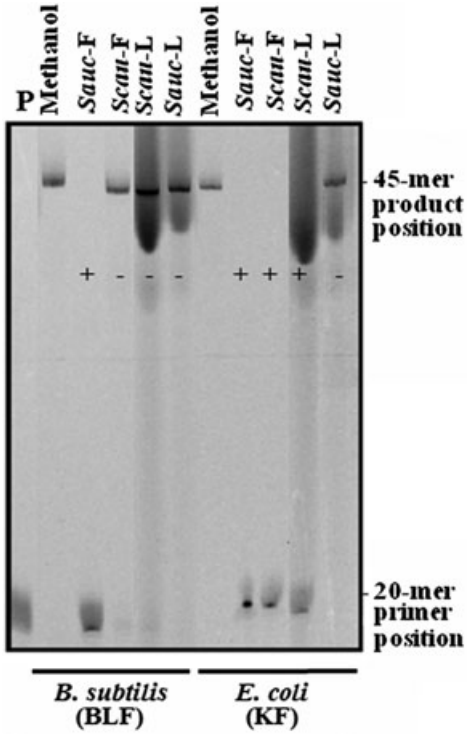

FIGURE 3 Effects of methanol extracts of two *Sorbus* species on nonreplicative DNA polymerases

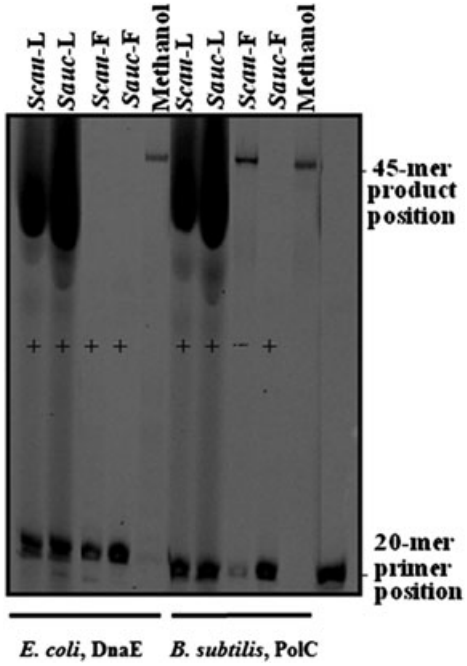

FIGURE 4 Effects of methanol extracts of two *Sorbus* species on replicative DNA polymerases

there was no polymerase activity at concentrations of 20% and above. In addition, ethyl acetate did not affect the polymerase activity at concentrations of 5%, but there was no polymerase activity at concentrations >10%. Methanol did not affect the polymerase activity of any polymerase at concentrations up to 20%. This is the first study to identify inhibitory effects of different organic solvents on bacterial polymerases.

Furthermore, different inhibitory effects were observed in leaf and fruit extracts of *Sorbus* species on replicative vs nonreplicative polymerase activity. While the fruit extract of *Sauc* inhibited both the KF and BLF, *Scau* only inhibited the KF but had no effect on BLF. Both fruit extracts of *Sorbus* had inhibited DnaE; *Sauc*, but not *Scau*, also inhibited polC. A strong inhibitory effect was observed in *Scau* leaf extracts against KF. Leaf extracts from both *Sorbus* species were unable to inhibit BLF activity, yet *Sauc* and *Scau* leaf extracts were able to strongly inhibit two replicative DNA polymerases (Figures 3 and 4). The development of resistance has rendered several antibiotics clinically ineffective, thus there is an urgent medical need for potent and safe antibacterial compounds with a novel mechansims of action. To generate new antibacterial drugs, the inhibition of DNA polymerase activity in living bacteria is considered an excellent therapeutic target. The *Sorbus* genus may be evaluated as a potential reservoir for inhibitory molecules against bacterial DNA polymerases.

3.3 | Antimicrobial activity

Inhibitory effects of plant extracts on microorganisms vary based upon the organism and chemical content of the extract. Extracts from *Sorbus* species displayed differing antibacterial activity against Gram-negative and Gram-positive microorganisms; as shown in Table 1, they were only able to inhibit the growth of Gram-negative organisms (Table 1). *Scau* leaf extracts inhibited the growth of *P. aeruginosa*, while it had no effect on *E. coli* or *S. typhimurium*. The fruit extract of *Sauc* had no effect on *S. typhimurium*, but it inhibited the growth of *E. coli* and *P. aeruginosa* (Table 1).

3.4 | The total phenolic content and DPPH radical scavenging activity of *Sorbus* extracts

The TPC of *Sorbus* extracts was calculated using the calibration curves of two representative phenolic standards: CGA, the most abundant cinnamic acid type phenolic compound in the extracts, and quercetin, a flavonoid. Leaf extracts had greater TPC than the corresponding fruit extracts, as revealed by phenolic profiling using HPLC-DAD (Table 4).

TABLE 4 Total phenolic content (TPC) and radical scavenging activities of the methanol extracts of two *Sorbus* species

	TPC		DPPH SC_{50}
Samples	(mg CGAE/g)[a]	(mgQE/g)[a]	(mg/mL)
*Sauc*F	22.843 ± 1.806	12.336 ± 1.010	0.366 ± 0.009
*Sauc*L	137.147 ± 8.248	75.549 ± 4.614	0.036 ± 0.001
*Scau*F	32.025 ± 0.768	17.128 ± 0.429	0.520 ± 0.023
*Scau*L	155.001 ± 10.884	85.658 ± 6.088	0.045 ± 0.000
Gallic acid			0.002 ± 0.000
Chlorogenic acid			0.006 ± 0.000
Quercetin			0.003 ± 0.000

CGAE, Chlorogenic acid equivalent; QE, quercetin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; SC_{50}, value of the concentration of sample required to scavenge 50% of DPPH radicals (mg sample per mL methanol).

[a]Total phenolics are expressed in mg CGAE/g dried extract and mg QE/g dried extract.

DPPH scavenging of the *Sauc* leaf extracts was the greatest of the extracts measured (0.036 mg/mL). *Scau* extracts had greater TPC than the *Sauc* extracts; DPPH radical cleavage activity correlated with the TPC ($r^2 = -0.94$).

4 | CONCLUSION

This study was the first to identify inhibitors of bacterial DNA polymerases in methanol extracts from the fruits and leaves of *Sorbus*. Searching the inhibitor agents in the fruits and leaves of *S. acuparia andS. caucasica* var. *yaltirikii*, a primer extension assay was used as a high-throughput method to identify inhibitors of DNA polymerases. Extracts were only able to inhibit the growth of Gram-negative bacterial microorganisms; therefore, *Sorbus* could be considered a potential source for Gram-negative specific antibacterial agents. In addition to their ability to inhibit DnaE and the KF, drugs derived from *Sorbus* extracts might provide selective inhibition of Gram-negative DNA polymerases. The ability of the extracts to selectively inhibit KF, BLF, DnaE and PolC may be caused by the phenolic compounds identified. The amount of phenolic compound and the DPPH scavenging activity were higher in the leaf extracts than fruit extracts. Leaf extracts primarily contained rutin derivatives while the fruit extracts mainly contained chlorogenic acid derivatives.

ACKNOWLEDGMENTS

This study was supported by a grant from The Scientific and Technical Research Council of Turkey (TUBİTAK-113Z054). We thank BioMed Proofreading LLC (USA) for providing a writing service.

REFERENCES

Ahmad, I., Mehmood, Z., & Mohammad, F. (1998). Screening of some Indian medicinal plants for their antimicrobial properties. *J Ethnopharmacol*, *62*(2), 183–193.

Aldasoro, J. J., Aedo, C., Navarro, C., & Garmendia, F. M. (1998). The genus *Sorbus* (Maloideae, Rosaceae) in Europe and in North Africa: morphological analysis and systematics. *Systematic Botany*, *23*(2), 189–212.

Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell*, *128*(6), 1037–1050.

Chakrabarti, R., & Schutt, C. E. (2001). The enhancement of PCR amplification by low molecular-weight sulfones. *Gene*, *274*(1–2), 293–298.

Chopra, I., Hodgson, J., Metcalf, B., & Poste, G. (1997). The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. *Antimicrobial Agents and Chemotherapy*, *41*(3), 497–503.

Coates, A., Hu, Y., Bax, R., & Page, C. (2002). The future challenges facing the development of new antimicrobial drugs. *Nature Reviews Drug Discovery*, *1*(11), 895–910.

Delarue, M., Poch, O., Tordo, N., Moras, D., & Argos, P. (1990). An attempt to unify the structure of polymerases. *Protein Engineering*, *3*(6), 461–467.

Devis, P. H. (2008). *Flora of Turkey and East Aegean Islands*. Edinburgh: University Press of Edinburgh.

Gil-Izquierdo, A., & Mellenthin, A. (2001). Identification and quantitation of flavonols in rowanberry (*Sorbus aucuparia* L.) juice. *European Food Research and Technology*, *213*, 12–17.

Goksin, A. (1977). A new variety of *Sorbus caucasica* Zinserl. var. yaltirikii Gökşin var. nova. *Biyoloji Dergisi*, *27*, 83–87.

Habich, D., & von Nussbaum, F. (2006). Platensimycin, a new antibiotic and 'superbug challenger' from nature. *ChemMedChem*, *1*(9), 951–954.

Hakkinen, S. H., Karenlampi, S. O., Heinonen, I. M., Mykkanen, H. M., & Torronen, A. R. (1999). Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *Journal of Agricultural and Food Chemistry*, *47*(6), 2274–2279.

Kenneth, K.-S. N., Arnold, J. J., & Cameron, C. E. (2008). In P. J. Paddison, & P. K. Vogt (Eds.), *Structure–Function Relationships Among RNA-Dependent RNA Polymerases*. (pp. 137–156). Heidelberg: Springer.

Kornberg, A., & Baker, T. A. (1992). *DNA Replication* (2nd ed.). New York: WH Freeman and Company.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology*, *26*(2), 211–219.

Olszewska, M. A., Presler, A., & Michel, P. (2012). profiling of phenolic compounds and antioxidant activity of dry extracts from the selected *Sorbus* species. *Molecules*, *17*(3), 3093–3113.

Projan, S. J. (2002). New (and not so new) antibacterial targets – from where and when will the novel drugs come?*Current Opinion in Pharmacology*, *2*(5), 513–522.

Ralser, M., Querfurth, R., Warnatz, H. J., Lehrach, H., Yaspo, M. L., & Krobitsch, S. (2006). An efficient and economic enhancer mix for PCR. *Biochemical and Biophysical Research Communications*, *347*(3), 747–751.

Raudone, L., Raudonis, R., Gaivelyte, K., Pukalskas, A., Viskelis, P., Venskutonis, P. R., & Janulis, V. (2015). Phytochemical and antioxidant profiles of leaves from different *Sorbus* L. species. *Natural Product Research*, *29*(3), 281–285.

Raudonis, R., Raudone, L., Gaivelyte, K., Viskelis, P., & Janulis, V. (2014). Phenolic and antioxidant profiles of rowan (*Sorbus* L.) fruits. *Natural Product Research*, *28*(16), 1231–1240.

Rothwell, P. J., & Waksman, G. (2005). Structure and mechanism of DNA polymerases. *Advances in Protein Chemistry*, *71*, 401–440.

Sandalli, C., Canakci, S., Demir, İ., Modak, M., & Belduz, A. (2010). Comparison of DIG-11-dUTP utilization by *Geobacillus caldoxylosilyticus* TK4, *Mycobacterium tuberculosis* and *Escherichia coli* DNA polymerases. *World Journal of Microbiology and Biotechnology*, *26*(3), 459–464.

Singleton, V. L. (1985). Citation classic – colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Current Contents/Agriculture Biology and Environmental Sciences*, *48*, 18.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature*, *406*, 775–778.

Wright, G. D. (2003). Mechanisms of resistance to antibiotics. *Current Opinion in Chemistry and Biology*, *7*, 563–569.

Wright, G. E., & Brown, N. C. (1999). Inhibitors of Bacillus subtilis DNA polymerase III. Structure-activity relationships of 6- (phenylhydrazino) uracils. *Curr Op Anti-Infect Invest Drugs*, *1*, 45.

Wu, S., Beard, W. A., Pedersen, L. G., & Wilson, S. H. (2014). Structural comparison of DNA polymerase architecture suggests a nucleotide gateway to the polymerase active site. *Chemistry Review*, *114*(5), 2759–2774.

How to cite this article: Turumtay H, Midilli A, Turumtay EA, et al. Gram (−) microorganisms DNA polymerase inhibition, antibacterial and chemical properties of fruit and leaf extracts of *Sorbus acuparia* and *Sorbus caucasica var. yaltirikii*. *Biomedical Chromatography*. 2017;31:e3901. https://doi.org/10.1002/bmc.3901