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Antibacterial Activities of *Kelussia odoratissima* and *Echinophora platyloba* Extracts and Essential Oils against Waterborne Pathogens

Running title: Antibacterial activities of *Kelussia odoratissima* Mozaff and *Echinophora platyloba* extracts and essential oils

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ABSTRACT

Natural antimicrobials, such as plant extracts (Es) and essential oils (EOs), are gaining popularity in food preservation. This study investigated the attendance of waterborne bacterial pathogens in drinking water samples (DWSs) from various urban centers in Shahrekord. It also examined the sensitivity of these bacteria to Es and EOs derived from *Kelussia odoratissima* Mozaff (KOME, KOMO) and *Echinophora platyloba* (EPE, EPO). In 2022, 200 distinct DWSs were gathered from various places in Shahrekord. *Escherichia coli*, *Salmonella typhimurium*, and *Helicobacter pylori* isolates were identified using routine biochemical and PCR tests, respectively. Following the preparation of hydroalcoholic Es and EOs, the chemical compounds were identified using the Folin-Ciocalteu method and gas chromatography-mass spectrometry (GC-MS), respectively. Using agar diffusion and micro-dilution methods, the antibacterial properties of KOME, EPE, KOMO, and EPO were evaluated against the bacterial isolates from drinking water. The overall frequencies of *E. coli*, *S. typhimurium*, and *H. pylori* were 2.5% (n = 5/200), 1% (n = 2/200), and 2% (n = 2/200), respectively. The total phenol content of KOME and EPE was 92.23±4.1 and 243.6±14.3 mg GAE/g, respectively. 5-methylsalicylic acid (40.57%) and cyclopropane (1-methyl-1,2-propadienyl)- (37.28%) were detected as the highest compounds in KOMO and EPO. The MIC and MBC values for the tested bacteria ranged from 250 to 1500 µg/mL and 500 to 2000 µg/mL, respectively. The most significant inhibition zone was observed for KOMO against *S. typhimurium* (20.03 ± 0.08 mm). KOMO and EPO had more robust antibacterial properties than EPE and KOME. The findings revealed the presence of pathogens in drinking fountains, posing potential health risks. Moreover, the results demonstrated the efficacy of EOs as potent antimicrobial agents, suggesting their promising applications for reducing pathogenic bacteria in the food industry.

Keywords: *E. platyloba, K. odoratissima* Mozaff, *E. coli*, *S. typhimurium*, *H. pylori*

INTRODUCTION

A drinking water distribution network (DWDN) is crucial in delivering reasonably safe drinking water to the populace. Despite the application of disinfectants to treat drinking water, which diminishes microbial growth, a diverse array of microorganisms persists within these distribution systems over time. Bacteria frequently accumulate inside water tubes, forming biofilms that serve as the origin of secondary bacterial pollution in these systems. These biofilms can have detrimental effects such as deterioration in water odor and taste, tube corrosion, decay of disinfectants, and the potential increase of waterborne diseases (1).

Ten main waterborne illnesses in developing countries contribute to over 28 billion diseases yearly. Microbiological examination is crucial for controlling the safety of different DWSs. The primary threat from microbes, particularly coliform bacteria, in water arises from contamination with human or animal excreta, highlighting the importance of ensuring safe drinking water sources. Coliform bacteria such as Klebsiella,

Salmonella, *Enterobacter aerogenes*, and *E. coli* can be found in water samples and are associated with various diseases including dysentery, cholera, bacillary dysentery, and typhoid in both livestock and humans (2).
Previous works have shown the presence of opportunistic pathogenic bacteria in various DWSs (3, 4). Al-sbaihy *et al*. (2024) conducted a microbial quality assessment of drinking water across twenty-one water sources classified into three levels in Libya. They identified six bacterial strains: *Pseudomonas aeruginosa, Cedecea lapagel*, *Ochrobacterum anthroi*, *Citrobacter freundii*, *Streptococcus anginosus*, and *Stenotrophomonas maltophilia* (5). Borjac *et al*. (2023) showed that bacteria isolated from household water reservoirs in Sidon, Lebanon, comprised intestinal *Enterococcus faecalis* (68%), *Staphylococcus aureus* (68%), and *P. aeruginosa* (22%) (6). Waterborne pathogenic bacteria also impact developed countries. In the United States, an estimated 560,000 people experience severe waterborne illness each year, with another 7.1 million suffering from mild to moderate infections, resulting in approximately 12,000 deaths per year (7). Over the decades, the misuse of antibiotics has emerged as a significant factor contributing to high antibiotic resistance among various pathogenic bacteria. *S. typhimurium* and *E. coli* are excellent models for studying the durability and transition of antibiotic-resistant bacteria through the food chain (8).
Many plants have been globally utilized as antimicrobial agents to control food spoilage microorganisms and food-borne pathogens. Plant Es and EOs comprise several bioactive compounds, including polyphenols, terpenes, and phytosterols (9, 10).
One of the newest genera in the Apiaceae family is the genus Kelussia. *K. odoratissima*, found exclusively in the central Zagros Mountains of western Iran, is known for its medicinal properties. It is traditionally used to treat ailments such as indigestion, rheumatism, gastric ulcer, cough, pain, and diabetes (11). Several studies have demonstrated that Es and EOs obtained from different parts of *K. odoratissima* exhibit diverse biological and pharmacological activities, including antioxidant, antibacterial, larvicidal, antileishmanial, cytotoxic, spasmolytic, anti-inflammatory, antihypertensive, sedative, and anxiolytic effects (12, 13). *E. platyloba*, a Umbelliferae family plant, is an antimicrobial agent for safeguarding food products from spoilage. In traditional medicine, it is used to strengthen the stomach. Furthermore, it is used traditionally as seasoning in yogurt and cheese (14). Numerous studies have confirmed the antimicrobial activity of EPE and EPO against a range of microorganisms, including Rhodotorula species, Candida species, *Listeria monocytogenes*, *E. coli*, *Bacillus subtilis*, *P. aeruginosa*, *Bacillus cereus*, *S. aureus*, and *Aspergillus niger* (15, 16).
Ensuring safe water access in all communities continues to pose a challenge for humanity. Novel approaches are required for effectively controlling pathogenic bacteria in water (17). Despite the relatively advanced stages of the Iran water-sector value chain, variations in potable water quality persist. Hence, this work aimed to explore the isolation of pathogenic bacteria in DWSs from various urban centers in Shahrekord and assess their sensitivities to KOME, EPE, KOMO, and EPO.

MATERIALS and METHODS

Sampling and Identification of Bacteria

This work was a cross-sectional study of 200 distinct DWSs gathered from various places in Shahrekord from the spring to summer of 2022. In the first step, Shahrekord was divided into five regions. Then, using the cluster sampling method, DWSs were gathered of water coolers, drinking fountains, and drinking water pipes situated in different settings, including parks (n=60), mosques (n=40), houses (n=40), and shops (n=60). The examination of DWSs commenced within 24 hours of collection, employing the most probable number (MPN) method along with confirmatory and supplementary techniques. Initially, the MPN was ascertained using Brilliant Green Bile Lactose Broth (BGLB, Merck, Germany), and tubes demonstrating growth were subsequently transferred onto MacConkey and eosin-methylene blue (EMB) agar plates (Merck, Germany). After an incubation period of 24 h at 44.5 ± 0.5°C, suspected colonies of *E. coli* were confirmed using SIM, TSI, and IMViC tests, following the protocols outlined by Nazemi *et al*. (18). To isolate Salmonella species, DWSs underwent filtration through a membrane with a pore size of 0.22 µm. Following this, 2 mL of buffered peptone water (BPW) was introduced to the filtered water and left to incubate at 25°C for four h. After pre-enrichment, 1 mL of BPW was removed and replaced with 1 mL of either 2 × Rappaport Vassilia (RV broth, Merck, Germany). Following incubation for 24 h at 43°C, a small loopful of each enrichment broth was streaked onto Tryptic Soy Brilliant Green-Sucrose

(TSBG-S) agar, and Xylose Lysine Decarboxylase-Novobiocin (XLDN) agar (Merck, Germany). Afterward, pink colonies with black centers (Salmonella-like colonies) were identified and transferred into TSI agar slants (Merck, Germany). Isolates that exhibited fermentation solely of glucose were then inoculated onto API 20E test strips (Analytab Products, USA) to identify Salmonella species (19). For the isolation of *H. pylori*, DWSs underwent filtration through 0.045 µm filter membranes. Subsequently, each membrane was submerged in 2 mL of Tryptic Soy Broth (TSB, Merck, Germany) for one h, after which 2 mL of TSB from each membrane was cultured. The samples were cultured on Brucella agar (Merck, Germany) supplemented with sheep blood (5%), colistin methanesulfonate (30 mg/L), nalidixic acid (30 mg/L), campylobacter selective supplement (5 mg/L), trimethoprim (30 mg/L), cycloheximide (100 mg/L), amphotericin B, vancomycin (10 mg/L), and fetal calf serum (7%). After a 72 h incubation period at 37°C under microaerophilic conditions, bacterial growth was examined and validated as *H. pylori* through gram staining, oxidase, and urease tests (20).

DNA Extraction and Bacterial Gene Amplification

The genomic DNA was extracted utilizing the phenol-chloroform method outlined by Raissy *et al*. (21). The concentration of the extracted DNA was determined using a NanoDrop spectrophotometer (Thermo Scientific, USA) at 260 nm and 280 nm wavelengths. The DNA concentration for the PCR reaction was standardized to 50 ng/µL (21). Additional information about the primer sequences can be found in Table 1.

Table 1 List of primers used for the detection of H. pylori, *S. typhimurium and E. coli*

Genes names	Primer sequence (5′-3′)	Size of product (bp)	References
UreB (H. pylori)	F: CTTAGCGTGGGTCCTGCTAC R: TGGTGGCACACCATAAGCAT	635	(22)
STM4497 (S. typhimurium)	F: CAGGTTCAGAGCCGCATTAGC R:GCCAGGCGTTACCCATTCC	360	(23)
rfbE (E. coli)	F: GTGCTTTTGATATTTTTCCGAGTAC R:TTTATATCACGAAAACGTGAAATTG	239	(24)

The PCR reaction was carried out using a PTC-100 thermocycler (Eppendorf, Germany) with a 50 µL reaction volume consisting of 2 µL of genomic DNA (at a concentration of 50 ng/µL), five µL of 10×PCR buffer (containing 100 mM Tris–HCl, pH 8.3, 60 mM MgCl2, 500 mM KCl, 1% Triton X-100, and 0.1% gelatin), one µL each of the one mM dNTPs, one µL of each primer (at a concentration of 50 pmol/µL), 0.2 µL units of Taq DNA polymerase (at a concentration of 5 units/µL), and 40 µL of sterile distilled water. The PCR condition was run under the following conditions: initial denaturation step for 2 minutes at 94°C; 25 cycles of denaturation for 1 minute at 92°C, annealing for 1 minute at 55°C, and extension for 90 seconds at 72°C; and a final extension step for 5 minutes at 72°C. The amplified products were separated via electrophoresis in agarose gels (1.5%) stained with ethidium bromide, running for 50 minutes at 90 volts. The gels were subsequently imaged utilizing a UV transilluminator. For the positive control, strains of *E. coli* (ATCC 87939), *H. pylori* (ATCC 43504), and *S. typhimurium* (ATCC 14028) were utilized.

Antimicrobial susceptibility test

Only bacteria confirmed by molecular methods (*S. typhimurium* and *E. coli*) underwent antimicrobial susceptibility testing against nine commonly used antibiotics, namely ceftriaxone (Cro) 30 µg, ampicillin (Amp) 30 µg, sulfamethoxazole/trimethoprim (Sxt) 22 µg, chloramphenicol (C) 30 µg, ofloxacin (Ofx) 5 µg, ciprofloxacin (Cip) 5 µg, tetracycline (Te) 30 µg, gentamicin (Cn) 10 µg, and erythromycin (E) 15 µg (HiMedia, India), following the disk diffusion method. Subsequently, the zone of growth inhibition (IZ) was compared with standards provided by the Clinical and Laboratory Standards Institute to identify the resistant isolates (25, 26).

Plant collection and preparation of hydroalcoholic Es and EOs

K. odoratissima and *E. platyloba* leaves were acquired from a spices and herbal market in Shahrekord, Iran. The dried leaves were ground into powder using a mixer grinder, after which each powder was extracted with 80% ethanol using the maceration technique. In summary, one hundred grams of each powder was mixed with 400 mL of ethanol (80%) and allowed to stand for 8 h at room temperature. The resulting Es were collected, filtered, concentrated, and stored under refrigeration (27). A Clevenger apparatus was used to prepare EOs from the plants.

Finally, the EOs were dehydrated using anhydrous Na2SO4 and stored in dark glass containers away from light at 4°C (28).

Determination of the Compounds by GC-MS and Colorimetric Methods

The chemical composition of the essential oils was analyzed utilizing a gas chromatograph system (Agilent 7890A, USA) fitted with an HP-5MS column (film thickness 0.25 µm, 0.2 mm × 50 m), coupled to a MS detector (Agilent Technologies, USA). The initial oven temperature was set to 40°C for 5 minutes, then increased to 125°C at a rate of 2°C/min, followed by a further increase from 125°C to 230°C at a rate of 5°C/min, and maintained at 230°C for 5 minutes. The flow rate of helium as the carrier gas was 1.0 mL/min. The volatile metabolites were identified by comparing their mass spectra and retention indices (RI) with the reference data available in the National Institute of Standards and Technology's (NIST) chemistry webbook library. Furthermore, the total phenolic constituents (TPC) in the Es were quantified using colorimetric methods outlined in a prior study. 20 mg of dried extracts was dissolved in 10 mL of 60% methanol. Then 100 µl of extract solution was added followed by 500 µl of 10% Folin Ciocalteu reagent and after 4 to 8 min were mixed with 400 µl of 7.5% aqueous sodium bicarbonate. The mixtures were allowed to stand for 30 min and the total phenols were determined by colorimetry at 765 nm with a spectrophotometer (Unico UV-2100, USA). The TPC of the Es was expressed as gallic acid equivalent (GAE) considering the equation of the prepared standard curve by the Folin-Ciocalteu reagent (27).

Antimicrobial properties of Es and EOs

Each bacterium (*S. typhimurium* and *E. coli*) was initially cultured in nutrient agar at 37°C for 18 h. The inoculum was then prepared by adjusting the concentration to half of the McFarland standard in a sterile saline solution, resulting in a concentration of approximately 1×10^8 CFU/mL (29). The growth medium for the cells was prepared by diluting a 5% v/v dimethyl sulfoxide (DMSO) solution in Mueller-Hinton broth (MHB). Subsequently, 95 µL of MHB and 5 µL of each isolate suspension were added into each well. Then, 100 µL of serial 2-fold dilutions of KOME, EPE, KOMO, and EPO were added to each well. The plates were placed in a horizontally shaking incubator and incubated at 37°C for 20 hours. Finally, the wells were examined for the presence or absence of bacterial growth based on turbidity. The MIC and MBC were defined as the lowest KOME, EPE, KOMO, and EPO concentrations that yielded no turbidity in the wells and no visible growth on the Mueller-Hinton Agar (MHA), respectively. The positive and negative controls consisted of wells containing MHB with and without bacteria.

The disc diffusion test followed the protocols outlined in the CLSI M02-A11 document (30). In summary, the aforementioned bacteria were spread onto MHA plates with a concentration of 1 × 108 CFU/mL. The blank discs (Sterile Whatman paper N.1, 6 mm) were employed for placing 50% concentrations of KOME, EPE, KOMO, and EPO, which were incubated at 37°C for 24 h. Afterward, the IZ diameters were assessed using a millimeter-scale ruler. Ampicillin, gentamicin, chloramphenicol, and DMSO 5% were employed as the positive and negative controls, respectively (10).

Statistical analysis

SPSS 14 was used for statistical analysis of antibacterial properties. Descriptive statistics were used to determine the relationships between the prevalence of bacteria and the variables such as kind of samples, season, and area. All interventional experiments were conducted in triplicate, and the resulting data were analyzed using Analysis of Variance (ANOVA). $P < 0.05$ indicated a statistically significant difference.

RESULTS

Bacterial Isolation

Five *E. coli* (2.5%), two *S. typhimurium* (1%), and two *H. pylori* (1%) strains were identified from a total of 200 distinct DWSs collected from various locations in Shahrekord (Table 2). Among these bacteria, 8.33% were isolated from DWSs collected from parks, while no bacteria were isolated from DWSs collected from houses. However, no *H. pylori* isolates were confirmed by PCR. The findings from their PCR analysis are illustrated in Figure 1.

All *E. coli* and *S. typhimurium* isolates also resisted tetracycline (Table 3).

Chemical Compounds of KOME, EPE, KOMO, and EPO

The total phenol content of KOME and EPE was determined to be 92.23±4.1 and 243.6±14.3 mg GAE/g, respectively. The volatile compounds present in KOMO and EPO and their chromatograms are shown in Table 4 and Figure 2. The primary chemical compounds identified in KOMO were 5-methylsalicylic acid (40.57%), and trans-2-methyl-3,8-oxomethano-bicyclo [6.4.0]undeca-4,6-diene (32.67%). EPO predominantly comprised cyclopropane, (1-methyl-1,2-propadienyl)- (37.28%), 2-fluoro-4,5-dicyanoimidazole (10.98%), and 3-benzofuranethanamine 5-methoxy (10.3%).

Antibacterial Activities of KOME, EPE, KOMO, and EPO

The IZ values, MIC, and MBC of KOME, EPE, KOMO, and EPO for antibacterial activities are provided in Tables 5 and 6. KOMO at a 50% concentration exhibited significantly higher IZ against the three mentioned bacterial strains than the other groups ($p < 0.05$). *S. typhimurium* was more sensitive to KOME, EPE, KOMO, and EPO than other bacteria ($p < 0.05$). The lowest MIC and MBC for KOME, EPE, KOMO, and EPO were also observed for ampicillin-resistant *E. coli*.

Table 2 The distribution of bacterial strains and their respective genes in DWSs across various locations

Specimens	Bacterial strains					
	E. coli		*S. typhimurium*		*H. pylori*	
	Conventional Tests	PCR Assay	Conventional Tests	PCR Assay	Conventional Tests	PCR Assay
Parks (n=60)	2 (3.33%)	2	1 (1.66%)	1	2 (3.33%)	0
Mosques (n=40)	1 (2.5%)	0	0	0	0	0
Houses (n=40)	0		0	0	0	0
Shops (n=60)	2 (3.33%)	0	1 (1.66%)	0	0	0
Total (n=200)	5 (2.5%)	2 (1%)	2 (1%)	1 (0.5%)	2 (1%)	0

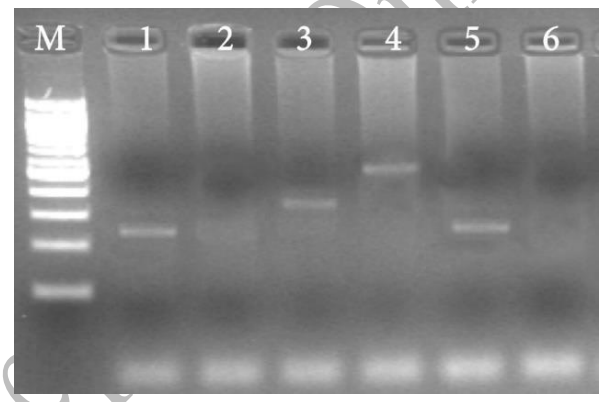

Fig. 1 PCR amplification of *UreB of H. pylori, STM4497* of *S. typhimurium*, and *rfbE* of *E. coli.* M: Marker (100 bp), lanes 1 and 2: positive samples of *E. coli* (239 bp), lane 3: positive sample of *S. typhimurium* (360 bp), lane 4: positive control of *H. pylori* (635 bp), lane 6: negative control.

Table 3 Antibiogram profiles of *S. typhimurium* and *E. coli* isolated from DWSs

Bacteria	Antibiotic								
	Amp	C	Cip	Cro	Cn	E	Ofx	Sxt	Te
S. typhimurium	S	S	S	S	S	S	S	S	R
E.coli	S	S	S	S	S	S	S	S	R
E.coli	R	S	S	S	S	R	S	S	R

Sensitive=S, Resistant=R, Ampicillin=Amp, Chloramphenicol=C, Ciprofloxacin=Cip, Ceftriaxone=Cro, Gentamicin=Cn, Erythromycin=E, Ofloxacin =Ofx, Suphamethoxazole/Trimethoprim=Sxt, Tetracycline=Te.

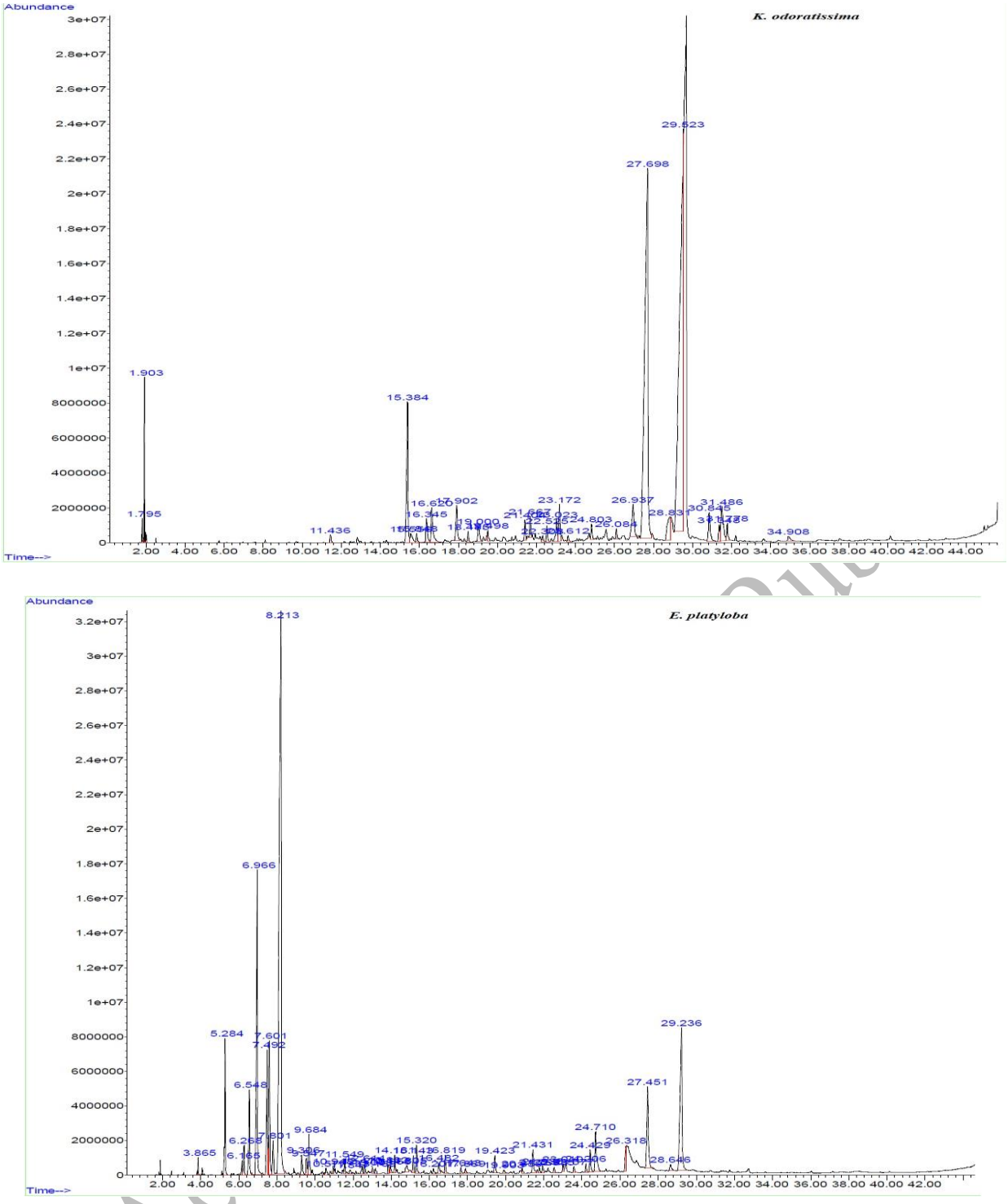

Fig. 2 GS-MS chromatograms of *K. odoratissima* and *E. platyloba* essential oils

Table 4 Chemical composition (%) for *K. odoratissima* and *E. platyloba* essential oils obtained with GS-MS analysis

Compounds	RT (min)	*K. odoratissima*	*E. platyloba*
tetra hydro-3-methyl- furan	1.79	0.23	-
2-ethyl-2-methyloxirane	1.90	1.25	-
pyridine, 4-ethyl-	3.86	-	0.28
cyclohexene, 1-methyl-4-(1-methylethylidene)-	5.284	-	3.53
benzeneacetic acid	6.16	-	0.39
cyclopropane, (1-methyl-1,2-propadienyl)-	6.26	-	0.81
butanedinitrile	6.54	-	2.7
2-fluoro-4,5-dicyanoimidazole	6.96	-	10.98
deltacyclene	7.49	-	4.32
2-ethyl-1-octen-3-yne	7.60	-	5.18

7,8-epoxybicyclo[4.3.0]non-2-ene	7.80	-	1.17
cyclopropane, (1-methyl-1,2-propadienyl)-	8.21	-	37.28
3-methylenecyclohexene	9.30	-	0.71
3-cyclohex-1-enyl-prop-2-enal	9.54	-	0.82
limonene	9.68	-	1.43
3-methylene-1,6-heptadiene	11.43	0.26	-
1-octen-3-yne	11.54	-	0.52
pyridine, 2,4-dimethyl-	12.47	-	0.4
2-decyne	12.61	-	0.39
2-pentyn-1-ol	13.83	-	0.32
z-citral	14.18	-	0.64
e-citral	15.14	-	0.59
4-methyl-1,4-heptadiene	15.32	-	0.99
3-cyclopropenoic acid,-1-butyl, methyl ester	15.38	5.35	-
7-oxabicyclo [4.1.0] heptane 2-methylene	15.55	0.57	-
1-methylcycloheptanol	15.84	0.30	-
4,7-diaminobenzofurazan	16.34	0.85	-
3-(4-hydroxyphenyl)propionitrile	16.62	1.62	-
2-hydroxy-3-cyanopyridine	18.48	0.31	-
[١'٢'٤]triazolo[1,5-a]pyridine, 2-	19.00	0.76	-
-٢hydroxy-3-methoxyphenylacetonitrile	19.42	-	0.68
bicyclo[3.2.0]heptan-2-one	19.49	0.36	-
2-methoxypyrimidine	21.40	0.59	-
1-methyl-2,4,5-trioxoimidazolidine	21.43	-	1.06
cyclooctyne	21.66	0.55	-
p-cyclohexanedione dioxime	22.30	0.21	-
aromadendrene, dehydro-	22.52	0.53	-
benzene, 1,3,5-tris(1-methylethyl)	23.02	0.88	-
2-methoxy-3h-azepine	23.17	1.32	-
٢'٦-dimethylnaphthalene	23.61	0.23	-
bicyclo[4.3.1]dec-1(9)-ene	24.20	-	0.31
benzaldehyde	24.42	-	0.92
2-ethoxy-6-oxo-1,2-azaphosphinane 2-oxide	24.71	-	2.29
2-methoxy-3h-azepine	24.80	0.50	-
3-methylpyridine 1-oxide	26.08	0.29	-
1,7-octadien-3-ol, 3,7-dimethyl-	26.31	-	0.98
1,3-cyclododecadiene, (e,z)	26.93	1.77	-
1,4-naphthalenedione, 2-hydroxy-3-methyl-	27.45	-	4.38
trans-2-methyl-3,8-oxomethano-bicyclo [6.4.0]undeca-4,6-diene	27.69	32.67	-
pyrido[2,3-d]pyridazine	28.64	-	0.36
trans-1-methyl-3,8-oxomethanocyclo [6.3.0]undeca-4,6-diene	28.83	1.76	-
3-benzofuranethanamine 5-methoxy	29.23	-	10.03
5-methylsalicylic acid	29.52	40.57	-
5-amino-1,3,3-trimethyl-indolin-2-one	30.84	1.39	-
tricyclo[3.2.1.0(2,4)]octane, 3-methylene	31.34	0.52	-
pyridine, 4-methyl-, 1-oxide	31.48	2.11	-
bicyclo[2.2.1]heptane-2,5-dione	31.77	0.55	-
hexadecanoic acid	34.90	0.29	-
Total	-	98.59	94.46

Table 5 Inhibition zone diameter (IZ) identified by disc diffusion method with 50% concentration of *K. odoratissima* and *E. platyloba* extracts and essential oils against *S. typhimurium* and *E. coli* isolated of DWSs.

Bacteria	Inhibition zone diameter (mm)						
	KOME	KOMO	EPE	EPO	Cn	C	Amp
E. coli	11.18 ± 0.18 cA	16.18 ± 0.17 aA	9.10 ± 0.13 dA	12.16 ± 0.18 bA	21	19	R
*E. coli**	13.18 ± 0.17 cB	17.23 ± 0.14 aB	10.5 ± 0.15 dB	14.16 ± 0.11 bB	19	21	20
S. typhimurium	12.05 ± 0.12 dC	20.03 ± 0.08 aC	14.08 ± 0.1 cC	15.08 ± 0.13 bC	19	20	16

Inhibition zone diameter, values represent mean of three replicates ± SD. Distinct capital letters within a column denote significant differences ($p < 0.05$), while different lowercase letters within a row signify significant differences ($p < 0.05$). KOME=*K. odoratissima* Mozaff extract, EPE=*E. platyloba* extract, KOMO=*K. odoratissima* Mozaff essential oil, EPO=*E. platyloba* essential oil. Amp=ampicillin (25 µg), C=chloramphenicol (30 µg), Cn=gentamicin (5 µg). *E. coli** was resistant to ampicillin.

Table 6 Minimal Inhibitory Concentration (MIC, µg/mL) and Minimal Bacterial Concentration (MBC, µg/mL) of *K. odoratissima* and *E. platyloba* extracts and essential oils against *S. typhimurium* and *E. coli* isolated of DWSs.

Extracts/essential oils	*E. coli*		*E. coli**		*S. typhimurium*	
	MIC	MBC	MIC	MBC	MIC	MBC
KOME	500	1000	250	500	1000	1500
KOMO	250	500	250	500	500	1000
EPE	1000	1500	500	1000	1500	2000
EPO	500	1000	250	500	1000	1500

KOME=*K. odoratissima* Mozaff extract, EPE=*E. platyloba* extract, KOMO=*K. odoratissima* Mozaff essential oil, EPO=*E. platyloba* essential oil. *E. coli** was resistant to ampicillin.

DISCUSSION

Following recommendations outlined by the World Health Organization (WHO) and other research findings, drinking water must remain devoid of *E. coli*, Salmonella, and other pathogenic bacteria (1). Previous research has highlighted the potential contamination of various water samples with pathogenic bacteria. For instance, Hasanvand *et al*. (2023) observed that among the samples tested, three out of fifty isolated bacteria from tap water samples indicated the likely presence of viable *H. pylori*. This suggests that DWDN could be a potential pathway for transmitting *H. pylori* (31). Chen *et al*. (2019) collected twelve water samples, comprising 6 stagnant and six flowing, from a municipal DWDN in eastern China. All six of the stagnant water samples were contaminated with bacteria (ranging from 1.2×10^3 to 2.8×10^4 CFU/mL). Specifically, 2 out of 6 stagnant samples were contaminated for coliforms, 3 out of 6 for Salmonella, 2 out of 6 for Shigella, and none tested positive for Legionella or Vibrio (1). Another study assessing the microbiological quality of DWSs from different sources in Libya (2024) identified six isolated strains as *C. lapagel, C. freundii, O. anthroi, P. aeruginosa, S. maltophilia*, and *S. anginosus*. Confidence value identities for these strains were reported as 90%, 99%, 90%, 95%, 99%, and 91%, respectively (5). In comparison, our study found a lower prevalence rate of *E. coli*, *S. typhimurium*, and *H. pylori* strains.

Ke *et al*. (2024) illustrated that *Acinetobacter junii, A. calcoaceticus,* and *Achromobacter xylosoxidans* contained mobile genetic elements (MGEs) and various multidrug antibiotic resistance genes (ARGs) such as transposases, integrases, and recombinases. These findings suggest that these bacteria, abundant in disinfected water, could potentially serve as pathogens and pose health risks (4). Previous studies indicated multidrug resistance of various bacteria, such as *E. coli*, *S. typhimurium*, to azithromycin, tetracycline, erythromycin, oxytetracycline, ertapenem (32, 33), ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (8). In our study, three isolates exhibited sensitivity to chloramphenicol, ciprofloxacin, ceftriaxone, gentamicin, ofloxacin, and sulfamethoxazole/trimethoprim. However, all *E. coli* and *S. typhimurium* isolates showed resistance to tetracycline. It's worth noting that environmental factors such as temperature, longitude, pH, latitude, and chlorine levels may impact the prevalence of antibiotic-resistance genes by directly influencing MGEs and the diversity of microbial communities (4).

One of the objectives of our investigation was to assess the antibacterial activities of KOME, EPE, KOMO, and EPO against bacterial isolates from drinking water. According to our results, EOs and Es from these two plants

contained high levels of bioactive chemical compounds, such as polyphenols. The KOMO was notably abundant in 5-methylsalicylic acid, whereas EPO exhibited a high cyclopropane concentration (1-methyl-1,2-propadienyl). It's essential to note that the phenolic compounds in plant Es and EOs exhibit significant diversity, influenced by the plant species, their origin, and the method of production (28, 29). The KOMO exhibited remarkable efficacy against all tested bacteria. Both KOMO and KOME displayed superior antibacterial activity compared to EPE and EPO. Additionally, the EOs from both plants demonstrated better antibacterial activity than the Es (Tables 5 and 6). The largest inhibition zone was observed for KOMO against *S. typhimurium* (20.03 ± 0.08 mm). Consistent with our findings, Miraj *et al*. (2016) reported satisfactory antibacterial activity of the ethanolic extract of *K. odoratissima* against *S. aureus* and *B. subtilis* (34). Mahmoudi *et al*. (2014) similarly demonstrated that KOMO exhibited higher efficacy against food-borne bacteria, with MIC and MBC values ranging from 1250 to 10000 ppm. Another study reported that KOMO displayed the most potent antimicrobial activity against *S. aureus, L. monocytogenes,* and *B. cereus,* with MIC values of 0.31, 0.62 and 1.25 and mg/ml, respectively (35). In previous work, it was observed that EPE discs with a concentration of 250 mg/ml resulted in the largest IZs, measuring 26.11 ± 1.16, 21.23 ± 0.89, and 19.65 ± 0.60 in the *S. enteritidis*, *S. typhi*, and *S. choleraesuis* groups, respectively (36). Another study noted that the most pronounced inhibitory effect of *E. platyloba* was seen in the ethanolic Es, which exhibited significant inhibition against all bacteria, particularly against *S. aureus*. Additionally, the results revealed that the weakest inhibitory effect on *A. baumannii, E. coli, S. aureus, S. flexneri* and *E. faecalis* was observed for the dichloromethane extract (37). Fayyaz *et al*. (2015) demonstrated that EPO exhibited MIC values of 0.05, 0.05, 0.05, 0.2, and 0.1 mg/ml, respectively, for *B. subtilis, S. aureus, E. coli O157, A. niger*, and *L. monocytogenes*. Their findings suggested the potential application of EPO in the food industry to inhibit the growth of certain food-borne bacteria (38).

The presence of antimicrobial components like phenolic and flavonoid compounds can inhibit the ATPase activity and increase the permeability of bacterial cell membranes, thus enhancing the sensitivity of microorganisms to external substances. One suggested mechanism of phenolic acid activity involves a reduction in extracellular pH, as seen with chlorogenic and gallic acids. Phenolic acid compounds exhibit membrane-active properties against bacteria, leading to the leakage of cell constituents such as proteins, nucleic acids, phosphate, and potassium. These compounds act at cytoplasmic and membrane levels (39-41). Zhou *et al*., (2023) reported that flavonoids can destroy the cell wall and membrane, cause the loss of intracellular substance, cause DNA damage, and even lead to cell death (42). A previous study showed that lipophilicity is crucial for flavonoids against gram-positive and gram-negative bacteria. This likely involves damaging phospholipid bilayers, inhibiting the respiratory chain, or ATP synthesis (43).

CONCLUSION

Indeed, ensuring access to safe drinking water for all remains one of the paramount challenges of the 21st century. Our findings revealed the presence of *E. coli*, *S. typhimurium*, and *H. pylori* in drinking water, notably in drinking fountains in parks. Antibiotic resistance was observed in three of the isolates. Standard microbiological analysis of drinking water should encompass assessments for coliforms, particularly *E. coli* and Salmonella spp., employing culture-based methods. Our study demonstrated that KOMO exhibited a more substantial inhibitory effect against the three pathogenic bacteria. Hence, using Es and EOs from medicinal plants within the food sector not only aids in antimicrobial functions but also enhances pharmacological aspects such as serving as food antioxidants, promoting healthcare benefits, and extending the shelf life of food items while preserving their nutritional content. A limitation of our study is the small sample size of DWSs, which may have affected the statistical power and generalizability of our findings. Consequently, we propose that forthcoming research should delve into elucidating the antibacterial and antioxidant mechanisms of Es and EOs within food models.

Authors' Contributions

Z.Y., MJ. D. and R. SC, were accountable for investigation, visualization, methodology, and drafting the original manuscript. A. S., E. R. and R. SC., were in charge of visualization, data curation, formal analysis, and supervision. All authors contributed to writing, reviewing, and editing the manuscript.

Conflict of Interests

The authors declared that there are no political, personal, commercial, or any other potentially conflicting interests related to the submitted manuscript.

Data Availability

All the data is available in the manuscript file.

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