RESEARCH ARTICLE

Dispersion of *Listeria monocytogenes* Biofilms in Raw Milk by Chlorogenic Acid and Phloridzin as Phenolic Compounds Extracted from Apples

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ABSTRACT

The development and spread of biofilms in the dairy sector are problematic because they increase cross-contamination, which reduces product safety and shelf life. This study's goal was to use phenolic chemicals derived from apple fruit to limit the growth of biofilms. So, 12 *Listeria monocytogenes* isolates were isolated out of 40 samples of raw milk from buffalo, cows, sheep, and goats. Most of the strongest biofilm producers were isolated from sheep and gout. Water: methanol (20:60 v/v) for 20 minutes at 40°C resulted in the highest total phenol content values with 37 mg. The methanol extract of apples had significant quantities of chlorogenic acid and phloridzin by chromatographic analysis with HPLC. After 24 hours, the proportion of biofilm inhibition was 44 to 77%; the inhibitory activity grew as phenolic component concentration increased. The proportion of inhibition for *L. monocytogenes* isolates from sheep and goats was highest, whereas the percentage for isolates from cows was lowest.

Keywords: *Listeria monocytogenes,* Biofilm, Phenolic extract, Apples.

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INTRODUCTION

Worldwide, milk is an important part of the human diet, but it also provides a favorable environment for the growth of numerous germs, particularly dangerous bacteria.[1] Therefore, it is believed that the quality of milk is crucial to the health and welfare of a community. Furthermore, bacteria continued to be the cause of all dairy illnesses. *Salmonella, Listeria monocytogenes, Staphylococcus aureus, Campylobacter*, and *Yersinia* are pathogens involved in communicable diseases linked to milk drinking.[2,3]

One of the most significant microorganisms for public health worries that can contaminate milk is *L. monocytogenes*. The bacterium that causes listeriosis in humans is a gram-positive, facultative, intracellular, non-spore-forming, motile, rod-shaped organism. Listeriosis is characterized by gastroenteritis, meningitis, and meningoencephalitis septicemia, especially in those with weakened immune systems, such as the elderly, pregnant women, and young children.[4,5] The bacteria is common in nature and may thrive in a variety of unfavorable conditions, including low pH and temperature, high salt and bile concentrations, oxidative stress, carbon shortage, and others, making it a potential food hazard.[3,6]

The distribution of untreated manure for agricultural purposes is regarded as a primary risk factor for the transmission of *Listeria*, thereby contributing to the spread of foodborne disease.[7] Animals may become infected through the consumption of *Listeria*-contaminated food, poor-quality silage, pathogen inhalation, direct contact with the source, drinking water, feed components, soil in which fodder plants were grown, silage feeding, sawdust bedding, and farm yard manure.[5,8]

Apples are one of the most popular fruits, in part because of their numerous health benefits. They are a fantastic source of nutrients since they are rich in carbohydrates, pectin, dietary fiber, and minerals.[9] Additionally, apples have been noted as one of the primary dietary sources of antioxidants, namely phenolic compounds, such as flavonoids and phenolic acids, and they also have high antioxidant potential. The primary flavonoid chemical classes found in apple fruit include anthocyanins like

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cyanidin glycosides, dihydrochalcones like phloridzin, and flavonols such as quercetin glycosides, catechin, epicatechin, and procyanidins.[10] The main objective of this study was to create an effective and precise analytical extraction method for phenolic compounds and assess their activity in the inhibition of biofilm formation by *L. monocytogenes*.

MATERIALS AND METHODS

Optimization of Extraction Conditions

At extraction time (5–30 minutes) and temperature (40ºC), the impact of 1-g of dried and crushed apples with 18 mL of water: methanol (60:20–20:60, v/v) was evaluated.

Total Phenolic Content

The Folin-Ciocâlteu method was used to calculate the total phenolic content of apple extracts.[11] 100 mL of extract, 5 mL of double-distilled water, and 0.5 mL of the Folin-Cioclâteu reagent were used for this. Double-distilled water and 1.5 mL of Na2CO3 (5 g/L) were added in increments of 10 mL after the mixture had been agitated and allowed to stand for 3 minutes. The samples that were produced in this way were heated to 50°C (in a water bath) for 16 minutes before being cooled to room temperature. Using a spectrometer, their absorbance was measured at 765 nm in comparison to the control sample (double-distilled water). The calibration curve for GA was created for the range of 0.2–0.8 mg/mL to calculate the total amount of polyphenols. A typical GA solution containing 1-mg/mL was used to prepare the solutions in this concentration range.

Characterization of Phenols by HPLC Analysis

A Symmetry C18 column (4.6 × 150 mm, 3.5 µm) was used for the separation of phenolic compounds, which was done at 20°C. Solvent A (2.5% acetic acid, v/v) and Solvent B were the components of the mobile phase (acetonitrile).[12] By comparing retention times and spectra of phenolic compounds to standards, it was possible to identify the compounds (gallic acid, chlorogenic acid, catechin, phloridzin, and myricetin).

Samples Collection and Preparation

A total of 40 samples, including raw buffalo milk, cow milk, sheep milk, and goat milk, were gathered in Baghdad city from street vendors and flocks of free-raised sheep and goats. To check for the presence of *L. monocytogenes*, all samples were transported to the lab in an icebox at 4°C.

Isolation and Identification of *Listeria*

The *Listeria* pre-enrichment broth was used to dilute each sample before it was plated on the *Listeria* oxford agar surface.[13] For 48 hours, all plates were incubated at 37°C. For further morphological and biochemical identification, the generated colonies were purified on tryptone soy agar with yeast extract and cultured at 37°C for 24 to 48 hours.

Biofilm Formation by *L. monocytogenes*

To test the ability to produce biofilms, *L. monocytogenes* isolates were injected into tryptic soy broth (TSB), which contained 2% (w/w) glucose, and had been cultured overnight at 37°C on tryptic soy agar (TSA). Each isolate's culture supernatants were cultured at 37°C for 24 hours before being diluted 1: 200 in TSB. Transferring aliquots of the 200 µL bacterial solution were done using a 96-well polystyrene microtiter plate with a flat bottom. The absence of the bacterial suspension in the medium served as the negative control. Planktonic cells were removed from the plates by washing them three times in phosphate-buffered saline after they had been cultured at 37°C for 24 hours (PBS; pH 7.2). Biofilms were cleaned with water before using 200 µL of gram's crystal violet dye for 1-minute. After being colored, biofilms were rinsed with water and dried. The amount of crystal violet binding was calculated by destaining the biofilms in 200 µL of 33% acetic acid for 10 minutes and measuring the crystal violet solution's absorbance at 595 nm.[14] Three to five times each assay had a comparable dramatic drop in absorbance values, and the findings were averaged.

Inhibition of Biofilm and Adhesion by Phenolic Compounds

The extracted phenols were tested to see if they may inhibit the development of biofilm in the previously mentioned biofilm-producing bacteria. The cells were given time to form a biofilm after the phenols were introduced to the growth medium and the isolates were injected. Before being applied to the plate, bacterial suspensions were diluted with water as a control and various concentrations of pure phenols (75, 150, and 300 µg/mL). The plate was incubated at 37°C for 24 hours. Before being colored for one minute with 200 µL of gram's crystal violet, biofilms were thoroughly rinsed with water. After being colored, biofilms were rinsed with water and dried. The amount of crystal violet binding was calculated by destaining the biofilms in 200 µL of 33% acetic acid for 10 minutes and measuring the crystal violet solution's absorbance at 595 nm. Three to five times were added to each experiment, and the absorbance values dropped dramatically each time. The following equation,[15] was used to determine the proportion of phenols' biofilm inhibition for each hazardous bacterium:

Percentage of biofilm inhibition (%) = $[1- (\frac{A}{Ao})]\mathbf{X100}$: where A refers to the absorbance of the well with the extracted phenols and A_0 refer to the absorbance of the control well

RESULTS AND DISCUSSION

Optimization of Extraction using Methanol as Solvent

Figure 1 shows that the 20:60 (v/v) water: methanol for 20 minutes at 40°C resulted in the highest values for total phenols, with a total phenol of 37 mg.

The extraction of apple phenolic compounds was more successful with diluted methanol (85%); this demonstrated that the mono-solvent method is less effective than a combination of solvents and water.[16] According to[17] some phenolic compounds are found in nature as glycosides, and the presence of sugars increases the phenolic compounds' water solubility.

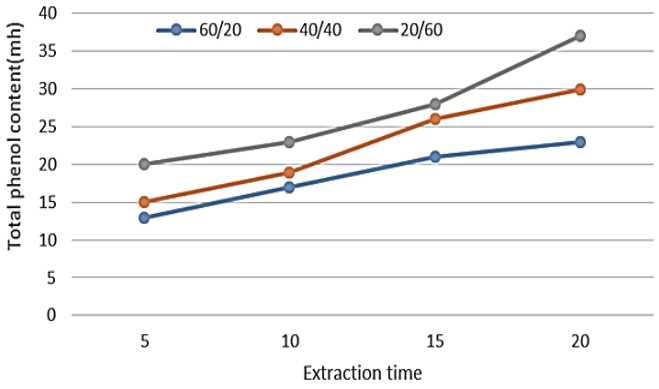

Figure 1: Effect of methanol and water ratios and different extraction times on total phenols content

Characterization of Phenolic Compounds by HPLC Analysis

The data in Figure 2 (a and b) demonstrated that the methanol extract of apples had significant quantities of chlorogenic acid and phloridzin compared to the commonly used standard phenolic compounds such as gallic acid, patechin, phloridzin, and myricetin.

Due to the phenolic chemicals present, apple pomace is an intriguing by-product. Phloridzin in particular, could be thought of as a biomarker to gauge the caliber of many apple products.[12] When extracting with methanol, chlorogenic acid and phloridzin had larger yields (32.4 and 48.4%, respectively).[18]

Isolation and Identification of *Listeria*

Twelve *L. monocytogenes* isolates were found in 40 samples of raw milk from buffalo, cows, sheep, and goats. As shown in Figure 3, the majority of *L. monocytogenes* isolates (5 isolates) were isolated from goat raw milk, followed by 3 isolates from sheep, 2 isolates from cow, and 2 isolates from buffalo.

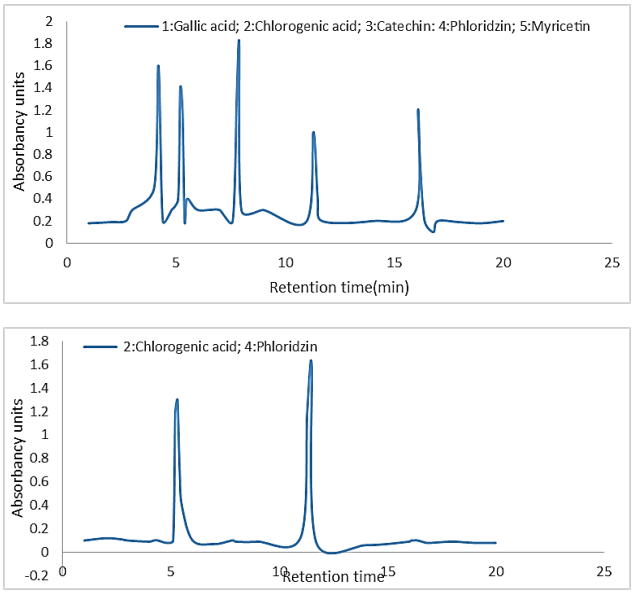

Figure 2: HPLC analysis for the phenolic extract from apples (a): standard sources of phenolic compounds; (b): apple extract

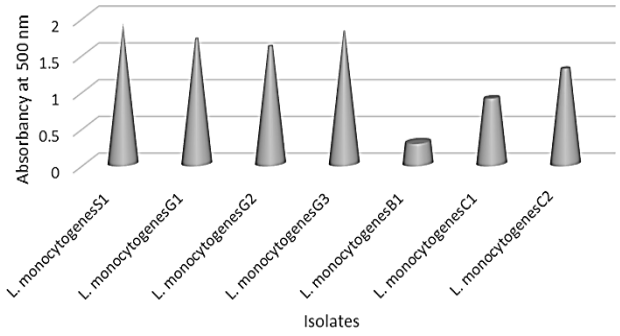

Figure 3: Biofilm formation by *L. monocytogenes* isolates

Highly perishable foods like milk and dairy products could be to blame for *L. monocytogenes* infections. Among the infections that are frequently spread by food in the USA, listeriosis is the third most prevalent cause of mortality.[19] Goat milk samples had the highest incidence of *L. monocytogenes* (26.7%), followed by buffalo milk and yogurt (13.3% each), kareish cheese (10%), and lastly sheep milk samples (6.7%).[20] The discovery of *L. monocytogenes* indicates that *L. monocytogenes* is present in raw milk contamination. The following risk factors have been linked to the need for *L. monocytogenes* contamination of raw milk: improper teat disinfection before milking, improper management of the barn and silage, inadequate hygiene practices in the environment, and unclean cows.[21] It has also been demonstrated that handling milk during storage and delivery contributes to milk contamination with different bacteria.[22]

Biofilm Formation by *L. monocytogenes*

The screening process for the determination of biofilm formers among *L. monocytogenes* isolates revealed that 7 isolates among *L. monocytogenes* isolates could produce biofilm. four isolates revealed strong biofilm production, 2 isolates were moderate and 1 isolate was a weak producer as in Figure 3. Most of the strongest biofilm producers were isolated from sheep and gout.

Among the isolates of *L. monocytogenes* strains isolated from meat products were more likely to be categorized as moderate or strong biofilm producers (57%) than bacteria derived from dairy products (28%).[23] In foodborne microbes like *L. monocytogenes*, the coordinated chemical signaling between bacteria has played a critical role in creating biofilms and structuring them biofilm.[24] The bacteria that induce a certain set of genes can detect and react to an increase in cell density. Some bacterial species may have their genetic materials expressed under the control of the quorum-sensing stimulus system, which increases the organism's capacity for resistance.[25]

Inhibition of Biofilm and Adhesion by Phenolic Compounds

The strongest and most commonly used *L. monocytogenes* isolates for biofilm generation were used to examine the effectiveness of phenolic compounds against biofilm growth.

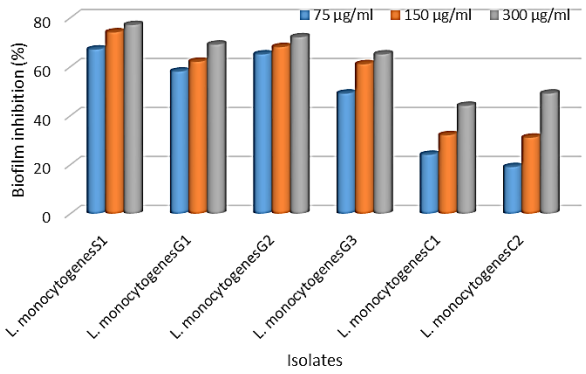

Figure 4: Effect of different concentrations of phenolic compounds on biofilm formation by *L. monocytogenes* isolates

All of the investigated *L. monocytogenes* isolates showed antibiofilm activity against phenolic compounds. According to Figure 4, the percentage of biofilm inhibition was 44–77% after 24 hours, and the inhibition activity grew as phenolic compound concentration increased. *L. monocytogenes* isolates from sheep and goats showed the highest percentage of inhibition, whereas isolates from cows showed the lowest percentage of inhibition.

Additionally, quercetin considerably decreased the number of bacterial cells that were still alive, broke up cell-to-cell connections, broke up established biofilms, and significantly decreased the expression of genes related to motility, virulence, and QS. Quercetin may be used as a replacement to regulate the *L. monocytogenes* biofilm in food systems and reduce the risk of foodborne diseases caused by this pathogen.[26] Food items (particularly beef and meat products) are inhibited by polyphenols under various storage settings, supporting their use as food preservatives against *L. monocytogenes*.[27,28]

CONCLUSION

The methanol extract of apples had significant quantities of chlorogenic acid and phloridzin. After 24 hours, the proportion of biofilm inhibition was 44–77%. The inhibitory activity grew as phenolic component concentration increased. The proportion of inhibition for *L. monocytogenes* isolates from sheep and goats was the highest, whereas the percentage for isolates from cows was the lowest.

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