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Original Article

Antimicrobial Resistance of Tannin Extract against *E. coli* Isolates from Sheep

Hamdi Abdulkareem, M[1 *], Abbas Abood, I[1], Munis Dakheel, M[2]

1. Microbiology Department, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
2. Zoonosis Research Unit, Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

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Corresponding Author: abcd@covm.uobaghdad.edu.iq

Abstract

Plants have been long valuable sources of natural materials that have served to preserve human and animal health; as a result, pharmacological purposes have arisen from the use of plant compounds in most countries, according to a World Health Organization report. The present study aimed to assess the antimicrobial resistance of tannin extract against *Escherichia coli* (*E. coli*) isolates in sheep. A total of 100 samples from sheep were used to isolate *E. coli* and treated with tannin extract (90% purity) to investigate the *in vitro* effect, as compared to some antibiotics (Clindamycin, Cephalexin, Kanamycin, Tetracycline, and Vancomycin). The bacterial samples were cultured in a selective and differential medium, and Gram staining was used to examine them. The biochemical assays were performed to purify and expose these cultures; moreover, the API 20E system and Rapid^{TM} ONE kits were utilized to confirm the bacterial strain. Based on the findings, 50% of the samples showed a positive result for the presence of *E. coli.* The well diffusion technique was used to investigate the antibacterial activity to confirm the antibacterial action of tannin extract (from pomegranate peel) in different concentrations against *E. coli*. The highest zone of inhibition for the bacteria ranged from 12±0.5 to 30.3±0.2 at 50% concentrations, proving that tannins extract was significantly effective against *E. coli*. The presence of *E. coli* was detected in 50 % of the samples. The well-diffusion technique was used to evaluate the antimicrobial property of tannin extract through various concentrations with the highest zone of inhibition for the bacteria ranging from 12.5 to 30.30.2 at 50%, demonstrating that tannin extract was significantly effective on *E. coli*.

Keywords: Antibacterial resistance, Antibiotics, *E. coli*, Pomegranate peel, Tannin extract

1. Introduction

Escherichia coli is a rod-shaped, facultative anaerobe, and Gram-negative bacterium which plays an important ecological role and might be used as a bio-indicator for antimicrobial resistance. It represents a normal flora in human and animal bodies, especially gastrointestinal tracts. Its genetic flexibility and adaptability to a constantly changing environment allow it to acquire a great number of antimicrobial resistance mechanisms. This bacterium has an ordinary relationship (commensalism) with its hosts, leading to infections in humans and animals in some circumstances. It is the most common human pathogen that causes serious diseases across the globe (1). The bacteria are commensal in the gut of humans and animals; nonetheless, they have complex strains that might transfer to pathogenic species. Moreover, the strains of *E. coli* could be classified into two major types. The first is the zoonotic-intestinal pathogenic *E. coli* and the second is extra-intestinal pathogenic *E. coli* demanding on their symptoms that reflected on their hosts and the kind of virulence factor expressed (2).

On the other hand, antibiotics are composed of chemicals with antimicrobial properties, demonstrating the ability to inhibit bacterial growth and cause cell death (3). They are globally used to prevent and treat humans and veterinary infectious diseases resulting from bacteria (4). Furthermore, bacteria might resist antibiotic action through the adaptation or the presence of resistance genes at the bacterial cells. Frequent exposure of bacteria to a sub-lethal antibiotics concentration may manifest an antibiotic transient resistance form (5). It was mentioned that antibiotic usage in animal husbandry, as growth promoters, would lead to an increase in bacterial resistance in animals, such as chickens (6). Resistance genes may play an important role in transferring antibiotic resistance from one bacterium to the other, leading to the development of antibiotic resistance in the environment since these genes are considered a mechanism for facilitating the spread among bacteria of the particular same species and also non-related bacteria (7).

Plants have been long valuable sources of natural materials that have served to preserve human and animal health; as a result, pharmacological purposes have arisen from the use of plant compounds in most countries, according to a World Health Organization report (8). Consequently, more than 90% of people in developed countries use traditional medicine to maintain their health and beauty; therefore, such plants should be examined for effectiveness and safety (9). Resistance is a global issue that is becoming more prevalent due to the widespread use of antimicrobial drugs; consequently, new materials derived from natural plants are being developed around the world to reduce the chance of the development of more antibiotic-resistant strains of bacteria and the formation of new harmful generations (10).

Pomegranate is mainly characterized by high tannin contents, which are responsible for effective antimicrobial properties (11). The pomegranate peels were used as a beneficial traditional medicinal plant in many areas as a remedy for dysentery and diarrhea (12). Moreover, the juice extract from pomegranate peel flour proved to have antimicrobial activity against many pathogenic bacterial strains, such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Listeria monocytogenes* (13). Tannin-rich plants, tannin-gallates, and gallic acid, which have been proven to have antibacterial properties, were shown to 51 certain bacteria and used in off-flavor-producing microorganisms in different studies (14).

The identification of the variables that influence tannins to inhibit bacteria and the methods by which bacteria overcome the inhibitory action would make for an improved gut ecosystem, reducing the anti-nutritional effects of tannin-rich plants and increasing animal production (15). In light of the aforementioned issues, the present study aimed to assess the antimicrobial resistance of tannin extract against *E. coli* isolates in sheep.

2. Materials and Methods

2.1 Animals of Experiment

A total of 100 samples were collected from local sheep (different areas in Baghdad/ Iraq) with no signs of illness.

2.2 Preparation of Tannin Extract

Tannin extract of pomegranate peel was obtained from local markets, Baghdad, Iraq. Thereafter, these samples were identified by the Department examination and certification of seeds, Baghdad, Iraq. Following that, the tannins were extracted and measured as described previously (11). The tannin in this material was 88% (standard error 5%), which was detected by HPLC/MS as described by Dakheel (16). Five concentrations of tannin were prepared by dissolving in distilled water v/v (10%, 20%, 30% 40%, and 50%). The dissolved extracts were used immediately after preparation.

2.3 Preparing Bacterial Strains

Escherichia coli was isolated and characterized biochemically and microscopically at the laboratories of the Microbiology department/ College of Veterinary Medicine/ University of Baghdad/ Iraq. All

of them were kept frozen in Tryptone Soya Broth (Himedia Laboratories Pvt, Ltd., Mumbai, India) and enriched with 30% glycerol until use. Before the application, *E. coli* was incubated in optimal conditions for its growth. Moreover, to prevent any interruption with any available natural acidity, the pH value was adjusted to 7.

2.4 Cultures and Microscopic Characteristics

After bacterial incubation, colonies were observed on nutrient, MacConkey, and Eosin-methylene Blue Agar (EMB agar plates. The suspected colonies were cultured using the streaking method. Sugar fermentation, peptone broth, raffinose, and arabinose were also applied to check an accurate identification and confirm the Gram-negative bacteria. The Gram staining was performed using a light microscope to differentiate between the strains of bacteria, followed by biochemical purification of the culture using API 20 E system and RapID ™ ONE System kits.

2.5. Determination of Antimicrobial Activity

The Mueller-Hinton agar (Himedia Laboratories Pvt, Ltd., Mumbai, India) was used for this test, and the use of the agar diffusion method depended on the initial inoculum which reached approximately 1.5×10^8 CFU/mL (0.5 McFarland scale) for the tested bacteria. In brief, an inoculum of one mL from *E.coli* was cultured at the plates on the surface of Mueller-Hinton agar, accompanied by the antibiotic discs as well. The antibiotics used in the current experiment were Clindamycin (DA 2 mcg/disc), Cephalexin (CL 30 µg/disc), Kanamycin (K 30 µg/disc), Oxacillin (OX 1 µg/disc), Tetracycline (TE 30 µg/disc), and Vancomycin (30µg/disc). They were served as reference antibiotics, while the tannin extract was tested by the addition of 10 µl/well to each petri dish that was incubated for 24 h at 37°C. Subsequently, the inhibition zone diameters were measured as triplicates (n=3), which were achieved for the same tannin extract and antibiotic discs.

2.6. Statistical Analysis

The data were analyzed in SPSS software using Duncan's multiple range test to compare the means (17).

3. Results

Plant extracts that were obtained from different sources, especially fruits, were studied for their antimicrobial activity. Tannins have been recognized as antimicrobial active substances; however, there are conflicting results (18). In the current study, the tannin aqueous extracts demonstrated satisfying results. As displayed in table 1, the respective inhibition zones were measured in (mm) and the results demonstrated that five different concentrations were used and gave significant outcomes. The first concentration was applied 10% (v/v) with a cutoff inhibition zone diameter of 12.0 ±0.5 mm, the second concentration was used 20% (v/v) with such a cutoff inhibition zone of 20.0 ±0.5 mm, the third concentration was 30% (v/v) with an inhibition zone of 25.5 ±0.6 mm, the fourth concentration was 40% (v/v) with an inhibition zone of 27±0.5 mm, and the fifth concentration was 40% (v/v) with an inhibition zone of 30.3 ±0.2 mm. It was revealed that 50% of the aqueous extract had the greatest inhibitory zone.

Table 1. Inhibition zones for different concentrations of tannin extract against *E. coli*

	The inhibition zone diameters (mm)				
	Concentrations of tannin extract				
	10%	**20%**	**30%**	**40%**	**50%**
E. coli	12.0 ± 0.5^{e}	25.5 ± 0.6^{d}	27.0 ± 0.5^{c}	28.3 ± 0.6^{b}	30.3 ± 0.3^{a}

Significant differences between concentrations at (P<0.01) showed as capital letters

4. Discussion

After incubation for 24 h, the results illustrated that the diameters of inhibition zones indicated *E. coli* sensitivity towards different concentrations of antibacterial substances with activities that were ocularly observed to be dependent on active material concentration. This finding is in agreement with the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006). Nonetheless, the highest inhibition zone diameter was 30.3 ±0.2 mm, which was recorded against the bacterium using the well diffusion method for tannin extracts from a low concentration to a high concentration in which the inhibition zone diameters proportionally increased during the use of 10%, 20%, 30%, 40%, and 50% against *E. coli*. This increase was significant (P<0.01). The lowest inhibition zone diameter for *E. coli* (12.0 ±0.5 mm) was recorded for extract at the lowest concentration of 10%. It is worth noting that bacterial resistance or susceptibility to certain antimicrobial agents would differ from various pathogens (19).

Furthermore, the results of the current study revealed that the widths of the zones of inhibition for Kanamycin (2 mm), Cephalexin (1.8 mm), Tetracycline (1.2 mm), Oxacillin (0 mm), Clindamycin (0 mm), and Vancomycin (0 mm) were the most efficient in the inhibition of *E. coli* by antibiotics. Daoutidou, Plessas (20) had previously observed a similar trend. When a bacterium is exposed to a sub-inhibitory dose of an antibiotic for an extended period, especially if the antibiotic is used in combination with other antibiotics, the corresponding genes of antibiotic resistance may develop. The antibiotic tetracycline is efficient; nonetheless, when it is administered to *E. coli*, it causes resistance to develop after long-term exposure to lower doses of a combination antibiotic (21).

The Kanamycin, on the other hand, was the most efficient among commonly used antibiotics in influencing *E. coli* as the largest inhibition zone, resulting in an insensitivity to the antibiotic when the inhibition zone diameter was greater than 30 mm and resistance when it was less. Oxacillin, Clindamycin, and Vancomycin all measured 0 mm, which might be related to *E. coli* status as a highly resistant Gram-negative bacterium that has grown more difficult to treat throughout the world (22). Therefore, it could be included in the ESKAPE pathogens group, which contains Gram-negative and Gram-positive bacteria, such as *Acinetobacter, Klebsiella, Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa* (23). Consequently, Kanamycin was the most effective antibiotic, with a smaller inhibition zone against *E. coli* using the well diffusion method than a 50% concentration of tannin extract. This finding led to the conclusion that tannins are effective natural substances against *E. coli* infections.

Authors' Contribution

Study concept and design: I. A. A.
Acquisition of data: M. H. A.
Analysis and interpretation of data: M. M. D.
Drafting of the manuscript: M. H. A.
Critical revision of the manuscript for important intellectual content: I. A. A.
Statistical analysis: M. H. A.
Administrative, technical, and material support: I. A. A.

Ethics

Ethical approval for the study was obtained from the University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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