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Article

Comparative Antibacterial Activity of Tannin-Enriched Aqueous Extracts of *Citrus limon* and *Capparis spinosa* Against Bacterial Isolates Recovered from Poultry Meat

Omar Ahmed Abdulqader
Omar.a.abdulqader@tu.edu.iq
Department of Biology, College of Education for Pure Sciences, Tikrit University, Tikrit, Iraq

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Abstract: The antibacterial activity of tannin extracts isolated from *Citrus limon* and *Capparis spinosa* against bacterial isolates recovered from poultry meat was studied in this research. VITEK 2 Compact system to determine eight bacterial species and agar well diffusion method on Mueller–Hinton agar at five extract concentrations (100, 75, 50, 25, and 12 mg/mL). Both extracts showed inhibitory activity, but *Citrus limon* consistently displayed a larger inhibition zone than *Capparis spinosa*. The maximum effective activity of *Citrus limon* was against *Providencia alcalifaciens* (27.23 ± 0.25 mm at 100 mg/mL), while the maximum effective activity of *Capparis spinosa* was observed against *Proteus mirabilis* (18.07 ± 0.12 mm at 100 mg/mL). Inhibition zone diameters were significantly influenced by extract type, concentration, and their interaction, as determined by statistical analyses. These results suggest that plant extracts, such as *Citrus limon*, which are high in tannins, can improve their viability and may be a potential natural antibacterial agent for the preservation of poultry. However, further studies (phytochemical characterization, MIC/MBC determination, and validation in real food storage conditions) are required to show the practical applicability of these substances in food safety systems.

Keywords: *Citrus limon*; *Capparis spinosa*; tannin extract; antibacterial activity; poultry meat; foodborne bacteria

Introduction

Poultry meat is one of the most important sources of animal protein in the world but it exhibits high susceptibility to microbial contamination during slaughtering, processing, handling, transportation and storage. Poultry meat is rich in nutrients and moisture, serves a good environment for the growth of spoilage and pathogenic bacteria, that is an important vehicle for foodborne microorganisms and remains persistent risk to the food safety [1], [2]. Poultry meat can be contaminated through several points in the food chain beginning from the intestinal microbiota of birds to processing equipment, workers, water, packaging surfaces and storage environments. This results in a wide

variety of bacterial species can be isolated from poultry meat, some related to spoilage while others with importance for public health. This scenario emphasizes that it is urgent to search for effective and safe antimicrobial alternatives to reduce bacterial counts and increase the microbiological quality of poultry products [1].

The increasing prevalence of the antimicrobial resistance and adverse effects associated with synthetic preservatives have prompted much research into natural food preservation methods in recent years. Plant-originating antimicrobials have gained great attention, as many plant materials are rich source of tannins, phenolic acids, flavonoids, alkaloids and essential oil constituents with wide spectrum of antimicrobial activity. Some of these compounds might work via multiple mechanisms, disrupting membrane stability, inhibiting enzyme activity, binding to cellular proteins and dysfunctionality in basic metabolic pathways [3], [4]. Research on the use of plant antimicrobials in food systems has gained interest, because these compounds may be effective in inhibiting microbial growth and, therefore may represent safer and more sustainable preservation strategies. Moreover, it has been stressed in recent reviews that plant extracts and their active compounds can also serve as a promising solution to improve food quality and safety; however, the antimicrobial effectiveness of such agents is governed by several factors including type of extraction method used, concentration employed, botanical source of the extract, target microorganism and food matrix traits [3].

Citrus limon is one of the mainly cited plant sources because it contains some important bioactive compounds, for instance its phenolic constituents and other phytochemicals similarly assist to antimicrobial activity. The antioxidant and antibacterial properties of bioactive compounds from different citrus fruits, which is why these materials have been progressively studied in food-related applications since they could serve as another source of natural preservatives. Consequently, Citrus limon is a promising candidate to study antibacterial activity on food-related microorganisms especially isolated from poultry meat [3], [5]. In a similar manner, Capparis spinosa is a medicinally and an aromatic plant which has been gaining the attention of scientists due to its diverse biological activities including antimicrobial activity. Previous findings demonstrated to us that crude extracts from Capparis spinosa (C. spinosa) can possess antimicrobial effects and contains several useful bioactive compounds, indicating its high potential for microbiological applications as well as food technologies. However, since its antibacterial effect differs depending on the plant part used, extraction procedure and bacterial species tested, it is necessary to further evaluate under clearly defined experimental conditions [6], [7].

Despite numerous experimental studies suggesting promising antimicrobial activity of plant extracts, their effects are not the same for all bacterial species. The structural differences of cell wall, membrane permeability barrier, existence or absence of intrinsic resistant mechanisms, and physical features can induce variation in susceptibility to plant-derived compounds. Thus, direct comparative studies with specific bacterial isolates and on strictly controlled concentrations are needed to ultimately ascertain which plant extract works better and against which bacterial species. These studies are more important when isolates come from food products of public health relevance such as poultry meat [1], [3]. The identification of bacterial isolates is also fundamental for the accurate analysis of antimicrobial results. Precise identification increases the scientific quality of comparisons and therefore enables sounder microbiological conclusions, since natural extracts can differ significantly in susceptibility between species. In the current study, the identification of bacterial isolates was performed with the widely used VITEK 2 Compact system for automated species-level identification of bacteria which provides confidence in validation of results produced in this work [8].

Therefore, this study aimed to evaluate the antibacterial activity of tannin extracts of Citrus limon and Capparis spinosa against bacterial isolates from poultry meat. Another objective of the study was to assess how different extract concentrations affected inhibition zone diameter, and to clarify if the two plants were dissimilar in their antibacterial activity against some bacterial species [3], [6].

Materials and Method

Study design

This laboratory-based experimental study was conducted to compare the antibacterial activity of tannin extracts obtained from Citrus limon and Capparis spinosa against bacterial isolates recovered from poultry meat. The inhibitory activity of each extract was assessed at five concentrations by measuring inhibition zone diameters around agar wells. All assays were performed in triplicate, and the results were expressed as mean ± standard deviation.

Source of isolates

Isolation of bacteria in the present study was performed from fresh and frozen poultry meat samples. The identification results indicated that the tested isolates included Staphylococcus lentus, Staphylococcus sciuri, Providencia alcalifaciens, Campylobacter jejuni species, Klebsiella pneumoniae, Proteus mirabilis Escherichia coli and Klebsiella oxytoca. These isolates were subsequently employed to assess the antibacterial activity of the evaluated tannin extracts.

Identification of isolates

Identification of the bacterial isolates was performed with the automated VITEK 2 Compact system (bioMérieux, France), based on biochemical reaction profiles. More detailed identification, such as species-level identification, has been performed using commercial biochemical tests, which have been time-consuming but shown inexpensive and acceptable accuracy results [8], [9].

Preparation of plant materials

Plant materials of Citrus limon and Capparis spinosa were cleaned, dried, and ground into fine powder. The powdered plant materials were then used for tannin extraction. Drying and grinding were performed to improve extraction efficiency and ensure homogeneity of the plant material before analysis.

Extraction of tannins

Extraction of tannins from dried plant powders was achieved using a modified aqueous extraction method described by Ahmed et al. Briefly, we boiled 50 mL distilled water with 0.5 g dried powder plant for 30 min. After that, the mixture was filtered through Whatman No. 1 filter paper and centrifuged at a speed of 2000 rpm for 20 min to obtain a supernatant. Tannins are separated according to their affinity for phenolic compounds (polyvinylpolypyrrolidone; PVPP was added). In order to eliminate excess impurities, the precipitated matter was washed before being collected and filtered several times with both ethanol and then ether. Subsequently, the crude tannin extract was dried and applied to the antibacterial assay [10].

Preparation of concentrations

In this study, two types of tannin extracts were selected: Citrus limon extract and Capparis spinosa extract. Each of the extracts was diluted at 5 concentrations (100, 75, 50, 25 and 12.5 mg/mL). These concentrations were chosen because they would establish the correlation between concentration of extract and antibacterial activity, plus it would allow us to compare both extracts when tested under identical conditions in the laboratory.

Preparation of inoculum

A fresh bacterial suspension was prepared from pure cultures of each organism, adjusted to 0.5 McFarland turbidity standard and inoculated on the agar plates. Standardisation of inoculum density is a critical requirement in diffusion-based susceptibility tests to allow consistent, comparable growth between bacterial strains permitting comparison of inhibition zone measures.

Antibacterial assay

The agar well diffusion method is among the most widely used techniques for screening antimicrobial activity against microorganisms with substances of plant origin [11]. Mueller-Hinton agar (MHA) was used as the culture medium as it is the recommended standard for diffusion-based antimicrobial susceptibility testing (CLSI, 2023). Confluent growth was obtained by spreading standardized bacterial inoculum over the surface of Mueller-Hinton agar plates with sterile swabs as per CLSI guidelines. With a sterile cork borer, aseptic wells of 8 mm diameter were made in the agar. Subsequently, 100 μL of each concentration was added in the appropriate well of every extract. Inoculated plates were incubated for 24 h at 37 °C, and after that the diameters of inhibition zones (in

millimeters) were measured. The agar well diffusion method offers an affordable option for comparing inhibition responses between different concentrations and bacterial types.

Controls

The negative control was deionized distilled water to verify that the inhibition observed was because of plant extracts and not due to the solvent. Ampicillin was used as a positive control to confirm the assay conditions and susceptibility of the isolates. The negative control had neither any inhibitory activity, while in the case of the positive control, clear zones of inhibition were formed.

Data collection

Inhibition zone diameters were measured as the mean of three independent replicates for each extract concentration and for each bacterial isolate. The measurements were taken in millimeters, and the data were shown as mean ± standard deviation for each treatment group. To make it suitable for comparison between bacterial species, extract types and concentrations, the values were organized into tables.

Statistical analysis

One-way analysis of variance (one-way ANOVA) was performed to examine statistically the effect of concentration upon inhibition zone diameter for each bacterial isolate and extract separately on experimental data. Bivariate correlation and two-way ANOVA statistical analyses were performed to assess the effect of extract type, concentration and interaction among all variables on antibacterial activity. Statistical significance was defined as a p-value < 0.05. Statistical outputs were described as F-values and their p-values.

Result and Discussion

Results

The susceptibility of eight bacterial isolates, recovered from poultry meat, to tannin extracts of Citrus limon and Capparis spinosa was determined using agar well diffusion method with a concentration range of 100, 75, 50,25 and 12.5 mg/mL. The isolates comprised Staphylococcus lentus, Staphylococcus sciuri, Providencia alcalifaciens, Campylobacter jejuni, Klebsiella pneumoniae Proteus mirabilis Escherichia coli and Klebsiella oxytoca. The inhibition zones were measured in triplicate and reported as mean ± standard deviation (Tables 2 & 3).

Table 1. Bacterial isolates recovered from poultry meat

No.	Bacterial isolate	Source of isolation
1	*Staphylococcus lentus*	Fresh chicken meat
2	*Staphylococcus sciuri*	Fresh chicken meat
3	*Providencia alcalifaciens*	Fresh chicken meat
4	*Campylobacter jejuni*	Fresh and frozen chicken meat
5	*Klebsiella pneumoniae*	Fresh and frozen chicken meat
6	*Proteus mirabilis*	Fresh and frozen chicken meat
7	*Escherichia coli*	Fresh and frozen chicken meat
8	*Klebsiella oxytoca*	Fresh chicken meat

Table 2. Effect of *Citrus limon* extract on inhibition zone diameter (mm)

Bacterial isolate	100 mg/mL	75 mg/mL	50 mg/mL	25 mg/mL	12.5 mg/mL
Staphylococcus lentus	19.53 ± 0.47	18.03 ± 0.55	15.07 ± 0.12	14.27 ± 0.25	12.13 ± 0.23
Staphylococcus sciuri	22.27 ± 0.31	17.27 ± 0.46	16.07 ± 0.12	14.33 ± 0.42	11.80 ± 0.35
Providencia alcalifaciens	27.23 ± 0.25	25.10 ± 0.17	25.35 ± 0.41	19.90 ± 0.17	15.13 ± 0.23
Campylobacter jejuni	18.37 ± 0.47	15.23 ± 0.40	13.27 ± 0.46	12.07 ± 0.12	11.17 ± 0.29
Klebsiella pneumoniae	24.90 ± 0.17	18.73 ± 0.25	19.33 ± 0.42	15.07 ± 0.12	12.60 ± 0.35
Proteus mirabilis	18.17 ± 0.29	15.20 ± 0.20	13.93 ± 0.12	12.17 ± 0.29	9.93 ± 0.12
Escherichia coli	16.73 ± 0.25	15.07 ± 0.12	14.67 ± 0.35	12.53 ± 0.50	0.00 ± 0.00
Klebsiella oxytoca	16.77 ± 0.25	15.00 ± 0.00	14.17 ± 0.29	12.93 ± 0.12	12.30 ± 0.26

Values are mean ± SD, n = 3.

Table 3. Effect of *Capparis spinosa* extract on inhibition zone diameter (mm)

Bacterial isolate	100 mg/mL	75 mg/mL	50 mg/mL	25 mg/mL	12.5 mg/mL
Staphylococcus lentus	13.90 ± 0.17	11.67 ± 0.29	10.83 ± 0.29	10.23 ± 0.40	9.93 ± 0.12
Staphylococcus sciuri	13.80 ± 0.20	12.73 ± 0.31	11.90 ± 0.17	10.13 ± 0.23	10.20 ± 0.35
Providencia alcalifaciens	17.07 ± 0.12	14.93 ± 0.12	15.33 ± 0.35	13.33 ± 0.29	13.83 ± 0.29
Campylobacter jejuni	13.23 ± 0.40	12.20 ± 0.20	10.90 ± 0.17	10.23 ± 0.40	0.00 ± 0.00
Klebsiella pneumoniae	15.00 ± 0.20	12.27 ± 0.23	12.23 ± 0.21	10.00 ± 0.00	10.33 ± 0.35
Proteus mirabilis	18.07 ± 0.12	14.90 ± 0.17	14.83 ± 0.15	13.20 ± 0.20	11.07 ± 0.12
Escherichia coli	14.63 ± 0.40	13.22 ± 0.23	11.73 ± 0.64	9.73 ± 0.46	9.90 ± 0.17
Klebsiella oxytoca	14.87 ± 0.23	12.67 ± 0.35	12.53 ± 0.50	12.13 ± 0.23	10.13 ± 0.23

Values are mean ± SD, n = 3.

Both of the plant extracts showed antibacterial activity against isolates tested but inhibition depended on species, extract type and concentration used. Overall, Citrus limon yielded a wider inhibition zone when compared to Capparis spinosa in the majority of bacterial isolates and concentrations. Both extracts exhibited a clear dose-dependent trend with the highest concentrations generally producing wider inhibition zones (Table 2 and Table 3).

For Citrus limon, the maximal inhibition zone was observed against Providencia alcalifaciens (27.23 ± 0.25 mm) at 100 mg/mL, followed by Klebsiella pneumoniae (24.90 ± 0.17 mm) and Staphylococcus sciuri (22.27 ± 0.31 mm), both also at the same concentration Escherichia coli was the least responsive organism with an inhibition percentage of 0.00 ± 0.00 mm at the lowest MIC (12.5 mg/mL). Progressive decreases of inhibition zones were observed for the other isolates (Table 2) — with measurable resulting inhibition even at lowest tested concentrations.

In Capparis spinosa, Proteus mirabilis at 100 mg/mL (18.07 ± 0.12 mm) and Providencia alcalifaciens at 100 mg/mL (17.07 ± 0.12 mm). The lowest concentrations show the weakest inhibition response with Campylobacter jejuni and Escherichia coli where no inhibition to minimal quantity depending on the isolate. In general, Capparis spinosa also showed antibacterial activity but the inhibition zones were smaller than those produced when using Citrus limon (Table 3).

One-way ANOVA showed that concentration had a significant effect on the diameters of inhibition zones for each isolate and each extract (all p-value < 0.0001). This lends support both to the potent concentration-dependent antibacterial action (Table 4). As can be seen from the two-way ANOVA, for most bacterial species, both type and concentration of extract had significant effects on inhibition zones and the interaction between extract type and concentration was also significant (indicated with asterisk number in Tables 2–5). This is an indication that the concentration effect may have been dependent on extract type. The only exception was for Escherichia coli, where the main effect of extract type was non-significant and concentration and the interaction term were highly significant (Table 5).

Table 4. One-way ANOVA summary for the effect of concentration on inhibition zone diameter

Bacterial isolate	Citrus limon F	Citrus limon p-value	Capparis spinosa F	Capparis spinosa p-value
Staphylococcus lentus	201.350	<0.0001	101.621	<0.0001
Staphylococcus sciuri	371.109	<0.0001	113.703	<0.0001
Providencia alcalifaciens	1071.123	<0.0001	100.368	<0.0001
Campylobacter jejuni	177.041	<0.0001	1075.853	<0.0001
Klebsiella pneumoniae	821.342	<0.0001	229.295	<0.0001
Proteus mirabilis	620.879	<0.0001	826.014	<0.0001
Escherichia coli	1513.824	<0.0001	77.128	<0.0001
Klebsiella oxytoca	202.790	<0.0001	79.379	<0.0001

Table 5. Two-way ANOVA summary for the effects of extract type, concentration, and interaction

Bacterial isolate	Extract F	Extract p-value	Concentration F	Concentration p-value	Interaction F	Interaction p-value
Staphylococcus lentus	1470.149	<0.0001	292.731	<0.0001	37.673	<0.0001
Staphylococcus sciuri	1665.688	<0.0001	463.842	<0.0001	95.933	<0.0001
Providencia alcalifaciens	6630.327	<0.0001	872.748	<0.0001	339.141	<0.0001
Campylobacter jejuni	1519.622	<0.0001	805.808	<0.0001	200.460	<0.0001
Klebsiella pneumoniae	4312.000	<0.0001	998.275	<0.0001	177.947	<0.0001
Proteus mirabilis	60.377	<0.0001	1361.566	<0.0001	19.528	<0.0001
Escherichia coli	0.106	0.7477	791.686	<0.0001	346.286	<0.0001
Klebsiella oxytoca	305.326	<0.0001	225.707	<0.0001	7.098	0.0010

Table 6. Control results

Control	Observation
Negative control (deionized distilled water)	No inhibition
Positive control (ampicillin)	Clear inhibition zones observed

Of the tested isolates, Providencia alcalifaciens was the most sensitive organism against Citrus limon while Proteus mirabilis was among the most sensitive for Capparis spinosa. On the other hand, Escherichia coli and Campylobacter jejuni were also sensitive to the plant-derived tannin extracts at lower concentrations (Table 2 and Table 3). Deionized distilled water was used as a negative control which did not show any inhibition, confirming that the antibacterial effect was due to the plant extracts. The inhibition zones observed in the positive control (ampicillin) were distinct, confirming the validity of the assay conditions (Table 6).

Discussion

The aim of the present study was to measure the antibacterial activity of tannin extracts obtained from Citrus limon and Capparis spinosa against semi-resistant bacterial isolates isolated from poultry meat. Nevertheless, concentrations and inhibition varied greatly between bacterial species and extract types. Conclusion: All in all, Citrus limon exhibited greater antibacterial activities than Capparis spinosa and the inhibition zones were consistently larger across most isolates. Both extracts demonstrated a strong dose-dependent relationship, whereby higher concentrations of the extract corresponded to increased inhibition confirming a plant-based antimicrobial concentration dependent mechanism. Citrus limon showed maximum inhibition against Providencia alcalifaciens (27.23 ± 0.25 mm at 100 mg/mL) while Capparis spinosa showed the best activity against Proteus mirabilis (18.07 ± 0.12 mm at 100 mg/mL). These results underscore important interspecies variability in susceptibility that may be accounted for by differences in bacterial cell envelope composition, permeability characteristics, and intrinsic resistance mechanisms. In contrast, at lower concentrations, Escherichia coli and Campylobacter jejuni demonstrated reduced sensitivity, including one condition where no inhibition of growth was observed in the presence of 10 μg/mL (but attained a much higher level (>25 μg/mL) under another condition;276 also indicating that bacterial physiology is key to susceptibility vs resistance.

The better efficiency of Citrus limon is also supported by its phytochemical profile (phenolic compounds, flavonoids and tannins are also known to damage membranes; inhibit enzyme activity; hinder metabolic processes necessary for life) [3], [5], [12], [13]. The biological plausibility of these findings is substantiated by earlier studies showing that postharvest microbial control by citrus-derived materials improved the quality of chicken meat. Conversely, the effect of Capparis spinosa matches the results previously published for its antimicrobial capacity but appears to have a weaker and extraction- and target bacteria-dependent effect [6], [14], [15].

Statistical analyses reinforced these observations. These findings were confirmed by a one-way ANOVA showing very significant impact of concentration on inhibition zone diameters against each

species, and two-way ANOVA also showing highly significant ($P < 0.001$) main effects of extract type and concentration and their interaction with most isolates tested, along with pairwise comparisons based on Tukey's post hoc test (data not shown). These results demonstrated a distinctive response in E. coli as compared to other organisms due to a lack of significant main effects and only interaction terms as the most influential factor.

The relevance of these results is underscored by poultry meat being a reservoir for various spoilage and pathogenic bacteria, which remain a challenge in terms of food safety [1]. It has been shown that Citrus limon tannin extract can also be applied as a natural antimicrobial but more detailed studies should be conducted to optimise its application within poultry preservation systems. Still, there are limitations of noting: for example; only inhibition zones were measured (not minimum inhibitory concentration – MIC or minimum bactericidal concentration - MBC); chemical composition of the extracts was not characterized and the assays conducted under in vitro conditions, which hardly mimic the complexity of actual food matrices [3], [11], [16].

Collectively, the results provide solid baseline data supporting concentration- and species-dependent inhibition of bacteria associated with poultry by tannin-rich extracts, particularly those from Citrus limon. These results warrant additional validation of the phytochemical characterization, MIC/MBC determination and practical application in relevant poultry meat preservation systems in food safety applications.

Conclusion

In our study, we confirmed that extracts of tannins from *Citrus limon* and *Capparis spinosa* had quantifiable antibacterial activity against bacterial isolates isolated from poultry meat; there was a strong interaction between extract type, concentration, and bacterial species. In conclusion, *Citrus limon* had better inhibition effect of all three bacteria on the percentage inhibition against *Providencia alcalifaciens* and *Capparis spinosa* had maximum activity against Proteus mirabilis. Both extracts exhibited a clear concentration-dependent response, with larger inhibition zones at higher concentrations confirmed by statistical analyses. The species-specific difference in susceptibility highlights the crucial role of bacterial physiology when examining antimicrobials from plants. Significantly, at lower concentrations *Escherichia coli* and *Campylobacter jejuni* exhibited reduced sensitivity including complete absence of inhibition under specific conditions indicating the limits in universal applicability.

Collectively, these data serve as compelling preliminary evidence for the potential of tannin-rich plant extracts, namely *Citrus limon* bark and leaf, to be developed as natural antibacterial candidates for use in poultry preservation systems. However, these extracts must still be investigated in terms of their phytochemical profile and established determining a minimum inhibitory and bactericidal concentration as well as carrying out studies at real food storage conditions. Such investigations will be critical for their regulatory applicability in food safety and sustainable preservation practices.

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