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ORIGINAL STUDY

Comparative Antibacterial Efficacy of Povidone-Iodine, Citric Acid, and Starch: A Study of Combined Activity and Interference Effects Against Clinically Relevant Bacteria

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ABSTRACT

Povidone-iodine and citric acid are widely used antimicrobial agents. However, data on their comparative efficacy and the functional outcomes of their combination, particularly regarding the influence of common excipients like starch, is limited. Understanding these interactions is crucial for optimizing antiseptic formulations. This study aimed to evaluate and compare the antibacterial activity of povidone-iodine, citric acid (5% and 20%), and starch (5% and 20%), both individually and in combination, against clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The antibacterial activity of the agents was assessed using an agar well diffusion assay. Mean inhibition zone diameters were measured and compared. The study design specifically controlled for dilution effects by dissolving solid citric acid and starch directly into the povidone-iodine solution for combination testing. Citric acid demonstrated potent, concentration-dependent antibacterial activity, yielding the largest inhibition zones (27.40 ± 1.54 mm for *S. aureus* with 20% CA). Povidone-iodine alone showed moderate activity. In combination, the antibacterial effect of the citric acid-iodine mixture was dictated by citric acid, with its efficacy fully preserved. In contrast, starch was inactive alone and its presence significantly reduced the antibacterial effect of povidone-iodine. *S. aureus* consistently showed the largest inhibition zones, while *E. coli* showed the smallest. Citric acid is a highly effective antibacterial agent whose activity is maintained in combination with povidone-iodine. Starch demonstrates a significant interference effect that reduces the efficacy of povidone-iodine, a finding not attributable to dilution. These results highlight the potential of citric acid as a primary antimicrobial and underscore the critical importance of ensuring physicochemical compatibility in antiseptic formulations.

Keywords: Povidone-iodine, Citric acid, Starch, Antibacterial activity, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa

1. Introduction

Bacterial infections and the widespread rise of antimicrobial resistance represent critical challenges to global public health, necessitating the continuous evaluation of effective antiseptic agents. Povidone-iodine (PVP-I) is a cornerstone of clinical antisepsis, demonstrating broad-spectrum antimicrobial efficacy in vitro and rapid bactericidal action against a range of clinically relevant pathogens. Its established mechanism involves the gradual release of free iodine, which disrupts microbial cell membranes and metabolic processes, leading to irreversible cellular damage [1]. The persistent need for potent

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and reliable antiseptics drives research into both established and alternative compounds.

Alongside traditional agents like PVP-I, organic acids have gained attention for their antimicrobial properties. Citric acid, a naturally occurring weak organic acid, has been reported to exert significant antibacterial effects by lowering pH, disrupting membrane integrity, and chelating essential metal ions, thereby inhibiting the growth of both Gram-positive and Gram-negative species [2]. In a different context, polysaccharides such as starch, while typically inert as antimicrobials, are often explored as carriers in composite materials. When combined with active agents, starch can be engineered to modulate stability and controlled release, although its intrinsic chemical properties can also lead to unintended interactions [3].

Despite extensive research on these agents individually, there is a notable gap in the literature regarding their comparative efficacy and interactive outcomes when combined. While the potent effects of citric acid are known, few systematic investigations have directly compared its antibacterial performance against a classic antiseptic like povidone-iodine in the same experimental setup [4]. Furthermore, the potential for formulation components like starch to interfere with or alter the efficacy of active agents such as iodine is a critical but often overlooked aspect of antiseptic development. Understanding these interactions is fundamental for creating stable and effective antimicrobial formulations.

Therefore, this study aimed to address these gaps by systematically evaluating the antibacterial activity of povidone-iodine, citric acid, and starch, both individually and in combination. The primary objectives were: (1) to compare the intrinsic antibacterial efficacy of these agents against clinically significant bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*); (2) to investigate the outcome of combining povidone-iodine with citric acid; and (3) to determine the effect of starch on the antibacterial performance of povidone-iodine. The findings are intended to provide clarity on the dominant active agents in these mixtures and to highlight potential interference effects relevant to antiseptic formulation.

2. Materials and methods

2.1. Study design and bacterial isolates

This experimental laboratory-based study was conducted to evaluate the antibacterial activity of povidone-iodine, citric acid (CA 5% and CA 20%), starch (S 5% and S 20%), and their combinations against clinically important bacterial pathogens. A total of 45 non-duplicate clinical bacterial isolates were included, comprising 15 *Staphylococcus aureus*, 15 *Escherichia coli*, and 15 *Pseudomonas aeruginosa*. The isolates were recovered from routine clinical specimens processed in the microbiology laboratory and identified using standard microbiological techniques, including colony morphology, Gram staining, and conventional biochemical tests. Identification was confirmed using the VITEk-2 system.

2.2. Preparation of bacterial inoculum

Each isolate was sub-cultured on nutrient agar and incubated at 37°C for 18–24 hours. Fresh colonies were suspended in sterile normal saline and adjusted to match the turbidity of a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL. This standardized suspension was used for all antibacterial susceptibility testing procedures.

2.3. Antimicrobial agents and test solutions

The agents evaluated were a commercial 7.5% povidone-iodine (PVP-I) solution, solid citric acid, and solid starch powder. A series of control and combination solutions were prepared for the assay. The control solutions included the commercial 7.5% PVP-I used as is, along with separate 5% and 20% (w/v) solutions of both citric acid and starch prepared in sterile distilled water. For the combination solutions, a method was employed to keep the PVP-I concentration constant and eliminate any potential dilution effects. Specifically, solid powders were dissolved directly into the 7.5% PVP-I solution. To create the citric acid combinations, 5 g and 20 g of solid citric acid were each dissolved into 100 mL of the PVP-I solution, yielding “PVP-I + CA 5%” and “PVP-I + CA 20%”. Similarly, the starch combinations were prepared by dissolving 5 g and 20 g of solid starch powder into 100 mL of the PVP-I solution to yield “PVP-I + S 5%” and “PVP-I + S 20%”. All solutions were freshly prepared and thoroughly mixed immediately before use. This methodology ensures that any observed changes in antibacterial activity within the combination groups are directly attributable to the interaction between the added substance and povidone-iodine, rather than to a dilution of the primary agent.

2.4. Agar well diffusion assay

Antibacterial activity was assessed using the agar well diffusion method on Mueller–Hinton agar plates. The standardized bacterial suspension was evenly

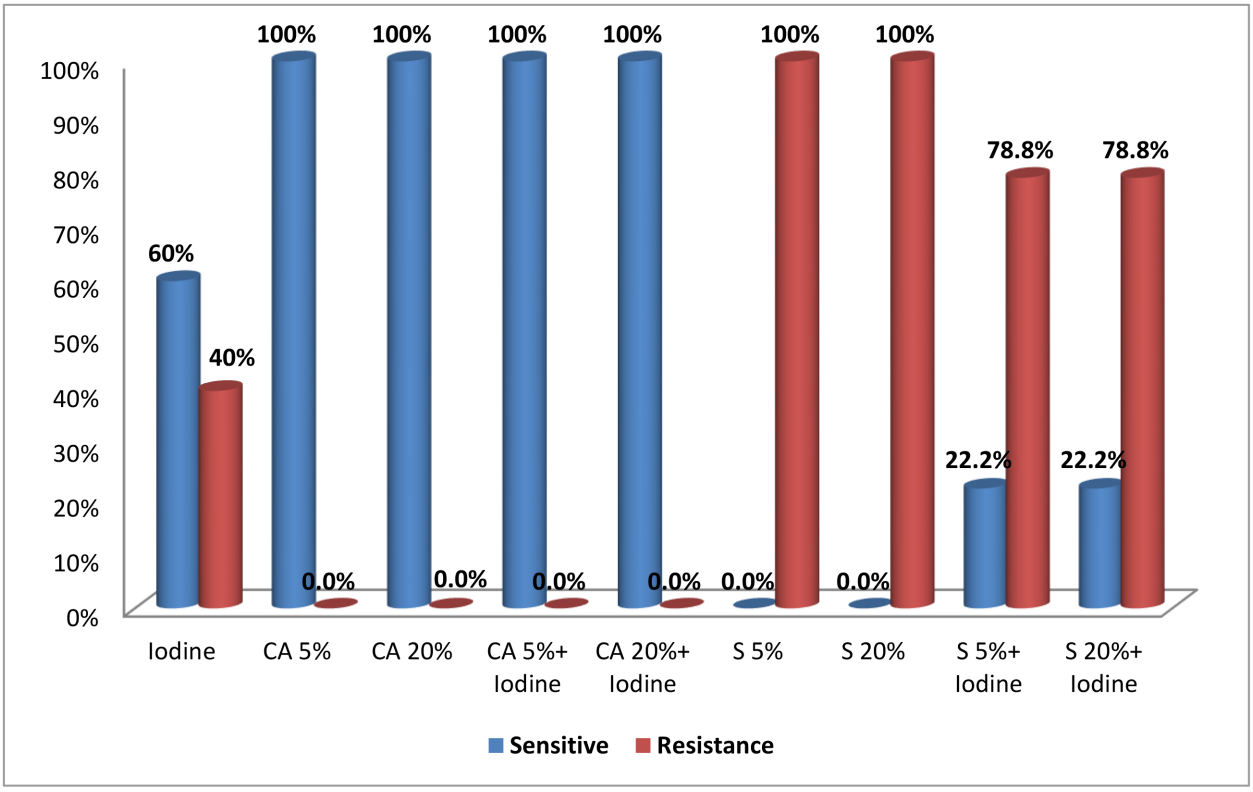


Fig. 1. The sensitivity patterns of bacterial isolates to povidone-iodine, citric acid, and their combinations. S= starch, CA= citric acid.

inoculated onto the agar surface using sterile cotton swabs to achieve confluent growth. Wells of 6 mm diameter were aseptically punched into the agar, and 100 μL of each test solution was dispensed into the wells. All plates were incubated aerobically at 37°C for 24 hours [5].

2.5. Measurement of antibacterial activity

After incubation, the diameter of inhibition zones around each well was measured in millimeters using a calibrated digital caliper. All measurements were performed in triplicate, and the mean value was recorded for each isolate–agent combination. Results were interpreted as sensitive or resistant based on the presence or absence of measurable inhibition zones.

2.6. Statistical analysis

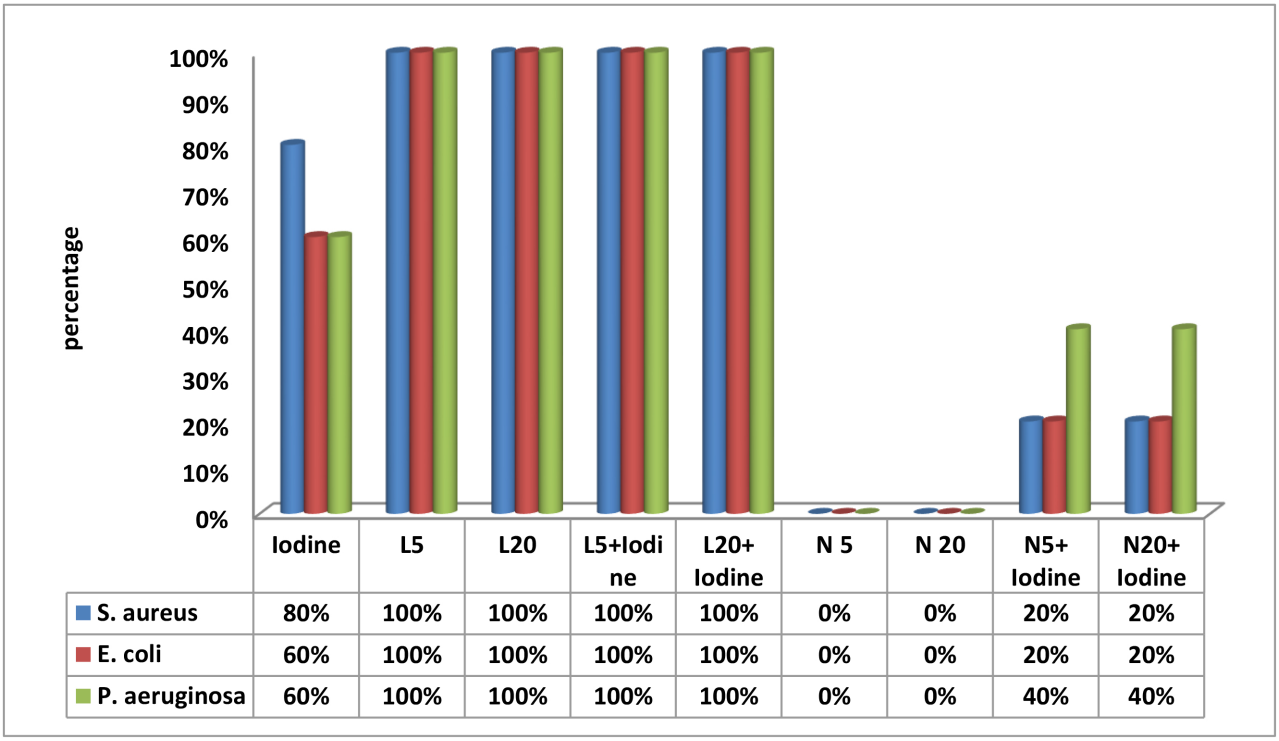
Data were analyzed using SPSS software (version 26). Descriptive statistics were calculated for all variables. Mean inhibition zone diameters were compared among bacterial species using one-way analysis of variance (ANOVA), and pairwise comparisons between antimicrobial agents were performed using independent sample t-tests. Pearson's correlation coefficient was used to evaluate the relationship between the antibacterial effects of different agents. A p-value < 0.05 was considered statistically significant.

3. Results

The antibacterial activity profiles of povidone-iodine, citric acid (CA), starch (S), and their combinations are summarized in [Fig. 1], showing the percentage of isolates that produced a measurable zone of inhibition. Povidone-iodine alone demonstrated moderate activity, with 60% of the tested isolates showing zones of inhibition.

In contrast, citric acid exhibited a strong antibacterial effect. At both 5% (CA 5%) and 20% (CA 20%) concentrations, all tested isolates (100%) produced zones of inhibition, indicating a potent and consistent inhibitory effect attributable to citric acid itself. When povidone-iodine was combined with either concentration of citric acid, inhibition was also observed in 100% of isolates, suggesting that the high level of activity seen with citric acid alone was maintained in the mixture.

Starch alone (at 5% and 20%) did not produce any measurable zones of inhibition (0% of isolates), indicating it lacks direct antibacterial activity against



	Iodine	L5	L20	L5+Iodine	L20+ Iodine	N 5	N 20	N5+ Iodine	N20+ Iodine
S. aureus	80%	100%	100%	100%	100%	0%	0%	20%	20%
E. coli	60%	100%	100%	100%	100%	0%	0%	20%	20%
P. aeruginosa	60%	100%	100%	100%	100%	0%	0%	40%	40%

Fig. 2. The sensitivity of bacterial isolates to different agents.

the tested organisms. However, when starch was combined with povidone-iodine, the percentage of isolates showing inhibition dropped markedly to 22.2%.

The percentage of isolates exhibiting zones of inhibition for each bacterial species is detailed in [Fig. 2]. When tested with povidone-iodine alone, inhibition was observed in 80% of *S. aureus* isolates and 60% of both *E. coli* and *P. aeruginosa* isolates.

Citric acid (at both 5% and 20%) and its combinations with iodine (CA 5% + iodine and CA 20% + iodine) resulted in measurable inhibition zones for all tested isolates (100%) across all three species (*S. aureus*, *E. coli*, and *P. aeruginosa*).

As expected, starch alone (S 5% and S 20%) produced no inhibition zones against any of the tested bacteria (0%). However, in the starch-iodine combinations, the percentage of isolates showing inhibition was substantially lower than with iodine alone. Specifically, inhibition was observed in only 20% of *S. aureus* and *E. coli* isolates, and 40% of *P. aeruginosa* isolates..

The mean diameters of the inhibition zones for each agent against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* are detailed in [Table 1].

Povidone-iodine alone produced the largest mean inhibition zone against *S. aureus* (18.00 ± 3.40 mm), followed by *P. aeruginosa* (14.60 ± 3.62 mm) and *E. coli* (12.40 ± 1.68 mm). The differences in zone diameters among the bacterial species were statistically significant ($p < 0.001$).

Citric acid at 5% (CA 5%) also produced the largest inhibition zone against *S. aureus* (18.80 ± 2.80 mm), with smaller zones for *P. aeruginosa* (17.40 ± 1.54 mm) and *E. coli* (13.80 ± 1.01 mm) ($p < 0.001$). When CA 5% was combined with iodine, the mean inhibition zones showed a slight increase for *S. aureus* (20.20 ± 3.23 mm) and *P. aeruginosa* (19.60 ± 3.24 mm).

At the higher 20% concentration (CA 20%), citric acid's antibacterial activity was markedly enhanced, yielding the largest inhibition zones observed in the study. The mean zone diameter was greatest for *S. aureus* (27.40 ± 1.54 mm), followed by *P. aeruginosa* (25.20 ± 0.77 mm), while *E. coli* showed a smaller zone (19.00 ± 3.33 mm) ($p < 0.001$). The addition of iodine to CA 20% did not result in a significant change in this activity, with nearly identical inhibition zones observed.

In stark contrast, the combinations of starch with iodine (S 5% + iodine and S 20% + iodine) yielded minimal to no inhibition zones. The mean zones were very small for *S. aureus* (2.60 ± 5.38 mm) and *E. coli* (2.80 ± 5.79 mm), and slightly larger but still limited for *P. aeruginosa* (8.20 ± 7.00 mm) ($p = 0.024$). These results quantify the substantial reduction in iodine's antibacterial effect when starch is present.

Table 1. The antibacterial inhibition zone activity of different agents.

Variables		No.	Mean	Std. Deviation	P value
Iodine	*S. aureus*	15	18.00	3.40	< 0.001
	E. coli	15	12.40	1.68	
	P. aeruginosa	15	14.60	3.62	
	Total	45	15.00	3.76	
Citric acid 5%	*S. aureus*	15	18.80	2.80	< 0.001
	E. coli	15	13.80	1.01	
	P. aeruginosa	15	17.40	1.54	
	Total	45	16.67	2.85	
Citric acid 5% + Iodine	*S. aureus*	15	20.20	3.23	< 0.001
	E. coli	15	15.40	3.31	
	P. aeruginosa	15	19.60	3.24	
	Total	45	18.40	3.85	
Citric acid 20%	*S. aureus*	15	27.40	1.54	< 0.001
	E. coli	15	19.00	3.33	
	P. aeruginosa	15	25.20	.77	
	Total	45	23.87	4.17	
Citric acid 20% + Iodine	*S. aureus*	15	27.20	1.52	< 0.001
	E. coli	15	18.80	3.66	
	P. aeruginosa	15	25.60	1.05	
	Total	45	23.87	4.35	
Starch 5% + Iodine	*S. aureus*	15	2.60	5.38	0.024
	E. coli	15	2.80	5.79	
	P. aeruginosa	15	8.20	7.00	
	Total	45	4.53	6.51	
Starch 20% + Iodine	*S. aureus*	15	2.60	5.38	0.024
	E. coli	15	2.80	5.79	
	P. aeruginosa	15	8.20	7.00	
	Total	45	4.53	6.51	

Across most treatments, *S. aureus* was the species with the largest inhibition zones, while *E. coli* consistently had the smallest, and *P. aeruginosa* showed an intermediate response.

Direct comparisons of the overall mean inhibition zones for each agent are presented in [Table 2]. The mean inhibition zone for citric acid at 5% (CA 5%) was 16.67 ± 2.85 mm, which was significantly larger than that of povidone-iodine alone (15.00 ± 3.76 mm, $p < 0.02$). Increasing the citric acid concentration to 20% (CA 20%) resulted in a substantially larger mean inhibition zone of 23.87 ± 4.17 mm, a significant increase compared to the CA 5% concentration ($p < 0.001$).

The combination of CA 5% with iodine yielded a mean inhibition zone of 18.40 ± 3.85 mm, which was significantly larger than that of CA 5% alone ($p = 0.017$). In contrast, combining CA 20% with iodine did not lead to any further increase in the mean inhibition zone, which remained at 23.87 ± 4.85 mm ($p = 1.0$).

Correlation analysis was performed to assess the statistical relationship between the inhibition zones produced by povidone-iodine and those from the two concentrations of citric acid [Table 3, Fig. 3]. A significant positive correlation was found between the inhibition zones of iodine and CA 5% ($r = 0.474$, $p = 0.001$). A stronger positive correlation was observed between iodine and CA 20% ($r = 0.603$, $p < 0.001$). As expected, a very strong positive correlation was found between the two citric acid concentrations, CA 5% and CA 20% ($r = 0.748$, $p < 0.001$), reflecting a consistent dose-dependent effect.

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4. Discussion

The results of the present study provide a comparative analysis of the antibacterial profiles of povidone-iodine, citric acid, and starch, both individually and in combination. A primary finding is the potent, concentration-dependent antibacterial activity of citric acid against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. This efficacy, which resulted in larger inhibition zones

Table 2. Comparison of mean of inhibition zone of different agents.

Groups	No.	Mean (mm)	Std. Deviation	P value
Iodine	45	15.00	3.76	< 0.02
Citric acid 5%	45	16.67	2.85	
Iodine	45	15.00	3.76	< 0.001
Citric acid 20%	45	23.87	4.17	
Citric acid 5%	45	16.67	2.85	< 0.001
Citric acid 20%	45	23.87	4.17	
Citric acid 5%	45	16.67	2.85	0.017
Citric acid 5% + Iodine	45	18.40	3.85	
Citric acid 20%	45	23.87	4.17	1
Citric acid 20%% + Iodine	45	23.87	4.85	

Table 3. Correlation between different agents.

Variables		Citric acid 5%	Citric acid 20%
Iodine	Correlation	0.474**	0.603**
	Sig.	0.001	0.000
Citric acid 5%	Correlation		0.748**
	Sig.	.	0.000

**Correlation is significant at the 0.01 level (2-tailed).

than povidone-iodine, is consistent with existing literature attributing its mechanism to cytoplasmic acidification, membrane disruption, and the chelation of essential metal ions critical for bacterial metabolism [6, 7]. The concentration-dependent activity observed aligns with the proton motive force dissipation model described for organic acids [8]. Povidone-iodine demonstrated moderate antibacterial activity, in line with its well-established role as a broad-spectrum antiseptic that functions by releasing free iodine [9]. Notably, when combined with citric acid, the antibacterial activity of the mixture was principally dictated by citric acid. While a modest increase in inhibition zone was observed at the lower 5% citric acid concentration, the effect at 20% was unchanged, suggesting the activity of citric acid is the dominant factor in these combinations rather than a synergistic interaction [10].

A critical finding of this study is the marked reduction in povidone-iodine's antibacterial effect when combined with starch. It is essential to clarify that this observation cannot be attributed to a dilution artifact, as solid starch was dissolved directly into the standard 7.5% povidone-iodine solution, maintaining a constant iodine concentration. This methodology strongly indicates a physical-chemical interaction rather than biological antagonism. The observed reduction in antibacterial efficacy is attributed to iodine sequestration within the amylose helices of starch [11, 12], a reversible physicochemical process

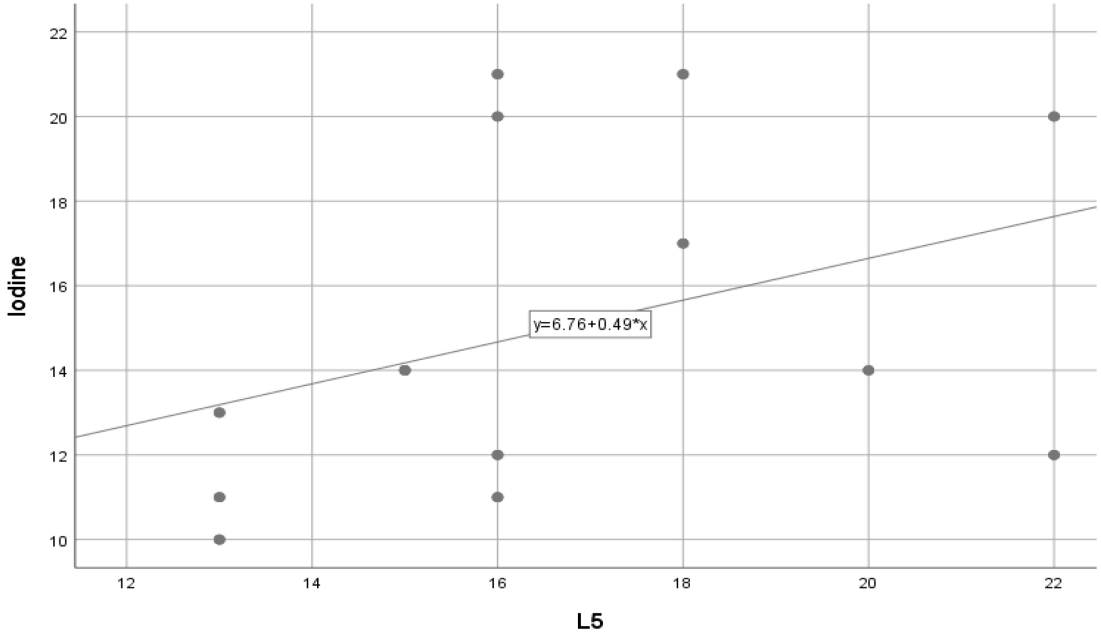

Fig. 3. Correlation analysis between Iodine and 5% citric acid (L5).

distinct from pharmacological antagonism. This phenomenon is analogous to the well-documented starch-iodine complex formation used in analytical chemistry [12], where iodine molecules become trapped within hydrophobic amylose helices, rendering them unavailable for microbial interaction. Importantly, this represents a formulation incompatibility rather than true antimicrobial antagonism [13].

This binding action reduces the bioavailability of iodine, limiting its ability to interact with and kill microbial cells. These results align with previous studies which report that unmodified polysaccharides can impair iodine's efficacy and underscore the need for chemical functionalization to create effective iodine-carrier complexes [11]. This highlights the critical importance of assessing excipient compatibility in antiseptic formulations, as chemically inert components can still produce significant negative functional outcomes [14].

The study also revealed species-dependent differences in susceptibility. *S. aureus*, a Gram-positive bacterium, consistently showed the largest inhibition zones, suggesting greater vulnerability to agents that target the cell wall and membrane. In contrast, *E. coli*, a Gram-negative bacterium, was the least affected, which may be attributed to the protective barrier of its outer membrane containing lipopolysaccharide and the presence of multidrug efflux pumps (e.g., AcrAB-TolC) that can limit the intracellular accumulation of antimicrobial compounds [15, 16]. The intermediate susceptibility of *P. aeruginosa* reflects its distinctive outer membrane porin profile and intrinsic resistance mechanisms [17, 18]. *P. aeruginosa* exhibited an intermediate response, consistent with its known intrinsic and adaptive resistance mechanisms that make it a challenging opportunistic pathogen [17].

It is important to acknowledge the limitations of this study. The antibacterial assessment relied on agar well diffusion, which does not distinguish between bactericidal and bacteriostatic effects and can be influenced by the diffusion kinetics of the agents. Future studies employing methods such as minimum inhibitory concentration (MIC) or time-kill assays would provide a more comprehensive understanding of these interactions. Additionally, while our methodology controlled for dilution, other physicochemical factors such as the pH and viscosity of the final solutions were not measured and could also influence outcomes. The statistical analysis utilized one-way ANOVA and t-tests; future work could benefit from repeated-measures models to better account for inter-isolate variability. Finally, the term "dominant effect" is used here based on observed inhibition zones and does not imply synergy, which would require formal testing via a checkerboard assay or calculation of the Fractional Inhibitory Concentration Index (FICI).

Despite these limitations, the findings have clear practical implications. The robust and stable antibacterial performance of citric acid, even in combination with iodine, positions it as a highly effective agent for clinical and industrial applications where a broad-spectrum, acidic antimicrobial is desired [19]. Conversely, the pronounced interference of starch with iodine serves as a critical cautionary tale for formulation science. It underscores that excipients must be selected not only for their physical properties but also for their chemical compatibility with active ingredients [20]. Future research should focus on exploring the precise molecular basis of these interactions and on designing functionalized carriers that can enhance, rather than hinder, the efficacy of established antiseptics.

It is important to clarify that the agar diffusion method, while semi-quantitative, was selected specifically for its suitability in detecting formulation interference effects. The complete abolition of iodine activity in starch combinations (evidenced by loss of inhibition zones despite constant iodine concentration) represents a robust qualitative endpoint that does not require MIC confirmation for validation. Nevertheless, we concur that broth microdilution MIC and time-kill assays would provide valuable supplementary data for future studies aimed at quantifying bactericidal kinetics [21].

5. Conclusion

In conclusion, this study demonstrates that citric acid exhibits potent, concentration-dependent antibacterial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*, and its efficacy is maintained when combined with povidone-iodine. In contrast, povidone-iodine shows moderate activity that is significantly diminished by the presence of starch, highlighting a critical interference effect. The findings confirm species-specific variations in susceptibility, with *S. aureus* showing the largest inhibition zones and *E. coli* the smallest. These results underscore the potential of citric acid as a primary antibacterial agent and emphasize the crucial need to ensure physicochemical compatibility between active ingredients and excipients in antiseptic formulations to avoid negative interactions.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of the Babylon Health Directorate, Ministry of Health, Iraq (Approval No.: BHD/EC/2025/017; Date of approval: 15/04/2025). As the study involved anonymized bacterial isolates collected as part of routine diagnostic procedures, no direct patient contact or identifiable personal data were involved.

Data availability

No datasets were generated or analyzed during the current study.

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Author contribution

Falah Hasan Obayes AL-Khikani conceived and designed the study; supervised the research project; contributed to methodology development; performed data analysis and interpretation; drafted the original manuscript; critically revised the manuscript for important intellectual content; and approved the final version for publication. **Mustafa Mutar Trrad** participated in laboratory experiments; contributed to bacterial isolation and identification procedures; assisted in data collection; and reviewed the manuscript. **Ali Abedulameer Alhusayni** contributed to sample processing; assisted in antimicrobial testing procedures; participated in data organization; and contributed to manuscript revision. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

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