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Research Article

Antibacterial Activity and Bioautography Test of Ethanol Extract of Kitolod (*Isotoma longiflora* (L.) C. Presl.) Leaves against *Staphylococcus aureus* and *Salmonella typhi* using Ultrasound-Assisted Extraction

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Abstract

Isotoma longiflora (L.) C. Presl. (Kitolod) is recognized for its potential as a natural antibacterial agent, with prior studies on its leaf extracts demonstrating inhibitory effects against *Staphylococcus aureus* and *Salmonella typhi*. This research aimed to investigate the antibacterial efficacy of *I. longiflora* leaf ethanol extracts, determine their phytochemical composition, and identify the active compounds responsible for the observed antibacterial activity. Extracts were prepared using ultrasonic-assisted extraction (34°C, 38 Hz, 40 minutes) with 70%, 85%, and 96% ethanol solvents. Antibacterial activity was assessed using the well diffusion method at concentrations of 40%, 60%, and 80%, against positive controls (ampicillin 10 µg and chloramphenicol 30 µg) and a negative control (100% DMSO). The highest activity against *S. aureus* was demonstrated by the 96% ethanol extract at 80% concentration, yielding an average inhibition zone of 13.3 ± 1.2 mm against *S. typhi*. The 85% ethanol extract at a concentration of 80% was most effective, with an inhibition zone of 9.5 ± 0.7 mm. Phytochemical screening confirmed the presence of alkaloids, flavonoids, saponins, and phenolics in both the 85% and 96% ethanol extracts. However, the attempt to identify the specific active antibacterial compounds via the contact bioautography method yielded negative results, suggesting the need for further isolation and identification studies.

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INTRODUCTION

The Kitolod (*Isotoma longiflora* (L.) C. Presl.) plant, native to the West Indies, is often regarded as a weed due to its prevalence in disturbed areas, such as ditch edges and damp, rocky locations. Nevertheless, this plant harbors significant potential as a traditional medicinal resource, with established ethnobotanical applications for treating eye pain, wounds, and sore throats, as well as documented anti-cancer and anti-inflammatory properties[1]. The global challenge of managing infectious diseases is complicated by the increasing prevalence of antibiotic resistance, which is fundamentally driven by the inappropriate use, unrestricted public access, and incorrect dosing of conventional antibiotics[2]. This critical public health issue highlights the need for ongoing research into novel antimicrobial agents[3].

Phytochemical analyses of *I. longiflora* leaves have consistently revealed the presence of important secondary metabolites. Studies by Wardani *et al.*[4] and Eff[5] reported that the ethanol extract of *I. longiflora* leaves contains key compounds, including alkaloids, flavonoids, saponins, tannins, and steroid triterpenoids. These constituents are likely responsible for the plant's

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documented bioactivities. Indeed, the ethanol extract of *I. longiflora* leaves has demonstrated notable antibacterial activity against clinically relevant pathogens, including *Staphylococcus aureus* and *Salmonella typhi*. For instance, a study using a 96% ethanol extract (maceration) showed inhibition zone diameters of 11.3 mm and 12.6 mm against *S. aureus* and *S. typhi*, respectively, at a concentration of 75%[6]. Further research confirmed significant activity, with a 96% ethanol maceration extract exhibiting the highest inhibition zone diameter against *S. aureus* at an average of 14.3 mm at a 300 mg/mL concentration[7].

In this context, efficient extraction methods are crucial for maximizing the yield and bioactivity of plant compounds. Ultrasonic-assisted extraction (UAE), which utilizes acoustic cavitation to form bubbles in the solvent spontaneously, is a superior technique compared to traditional maceration. This process mechanically damages the plant's cell walls, significantly enhancing solvent penetration and offering advantages such as increased extraction yields, lower operating temperatures, reduced solvent volumes, and shorter processing times[8]. This study employed the UAE method with varying ethanol solvent concentrations (70%, 85%, and 96%) to prepare the *I. longiflora* leaf extract.

The concentration of the solvent is a critical parameter that can profoundly influence the resulting extract's chemical profile and, consequently, its antibacterial efficacy. Previous research, such as that by Mubarak *et al*.[9] on bligo fruit (*Benincasa hispida* Thunb), demonstrated that varying ethanol concentrations (50%, 70%, and 96%) resulted in the highest anti-*S. typhi* activity at the 70% concentration (25.22 mm average inhibition zone diameter). Building upon this knowledge, the present study aims to evaluate and compare the antibacterial activity of 70%, 85%, and 96% ethanol extracts of *I. longiflora* leaves, prepared using the UAE method, against *S. aureus* and *S. typhi*. Furthermore, the researchers conduct a phytochemical screening on the prepared extracts to determine their chemical composition and subsequently attempt to identify the specific compounds that are responsible for the observed antibacterial effects.

MATERIALS AND METHODS

Materials

The study utilized a comprehensive set of laboratory equipment and high-purity chemical reagents. Essential instrumentation included a drying cabinet, sonicator (Branson), rotary evaporator (Stuart), water bath (Memmert), Laminar Air Flow (LAF), oven (Memmert), autoclave (Hirayama), incubator (Memmert), and a micropipette (Socorex). General laboratory supplies comprised chambers, Petri dishes, a glass spreader, cork borers, test tubes, and a UV lamp (254 and 366 nm).

The plant material, *I. longiflora*, was collected from the Tulung subdistrict. Its taxonomic identity was formally confirmed at the FKIP Biology laboratory at Universitas Muhammadiyah Surakarta. This determination step was crucial for ensuring the accuracy and minimizing the risk of contamination or misidentification of the research material. The determination letter (No. 007/A.E-I/LAB.BIO/V/2023) certified the plant as *I. longiflora* (L.) C. Presl., belonging to the family *Campanulaceae*.

Chemical reagents and media used included 70%, 85%, and 96% ethanol, distilled water (technical grade), 100% dimethyl sulfoxide (DMSO; pro analysis grade), and various solvents: *n*-hexane (pro analysis grade) and ethyl acetate (pro analysis grade). For qualitative phytochemical screening, the following reagents were employed: 2 N HCl (pro analysis), magnesium powder, concentrated HCl, Dragendorff reagent (pro analysis), cytroborate reagent (pro analysis), and 10% $FeCl_3$ reagent (pro analysis). Chromatographic separation was performed using GF_{254} silica gel plates. Microbiological studies required Mueller-Hinton Agar (MHA) and Brain Heart Infusion (BHI) media, along with commercially available antibiotic discs (OxoidTM): Ampicillin 10 µg, Erythromycin 15 µg, Chloramphenicol 30 µg, Tetracycline 30 µg, and Vancomycin 30 µg.

Methods

Ultrasonic-assisted extraction

The UAE process was initiated by accurately weighing 50 g of the prepared *I. longiflora* leaf simplisia. This material was then separately macerated in 70%, 85%, and 96% ethanol solvents, maintaining a fixed simplicia-to-solvent ratio of 1:10 (w/v). Each mixture was subjected to sonication in an ultrasonic bath for 40 minutes at a controlled temperature of 34°C and a frequency of 38 Hz. Following the initial extraction, the resulting filtrate was collected, and the residual plant material was subjected to a re-extraction using fresh portions of the corresponding ethanol solvents to maximize compound recovery.

The extracts from both the initial and re-extraction steps were combined and subsequently concentrated in vacuo using a rotary evaporator at 50°C and 80 rpm to remove the majority of the solvent. Final concentration to a viscous, thick consistency was achieved by heating the semi-solid extract on a water bath. The resulting extraction yield was calculated gravimetrically using the following **Equation 1**:

$$\text{Yield (\%)} = \frac{\text{Weight of concentrated extract}}{\text{Weight of initial simplicia}} \times 100\% \qquad [1]$$

Bacterial sensitivity test

The bacterial sensitivity assay commenced by dispensing 200 µL of the standardized test bacterial suspension onto the surface of MHA plates. This inoculum was evenly distributed across the entire surface using a sterile glass spreader and allowed to dry briefly. Subsequently, pre-prepared antibiotic discs were precisely positioned onto the inoculated MHA media. For *S. aureus*, the antibiotic discs tested included Ampicillin (10 µg), Chloramphenicol (30 µg), Tetracycline (30 µg), and Vancomycin (30 µg). Conversely, the sensitivity of *S. typhi* was evaluated using discs containing Ampicillin (10 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), and Vancomycin (30 µg). The plates were then incubated at 37°C for 24 hours. Following incubation, the diameter of the zone of inhibition surrounding each antibiotic disc was meticulously measured. The antibiotic that demonstrated the largest inhibition zone diameter for each respective bacterial strain was subsequently designated as the positive control for the assay.

Antibacterial activity test

The antibacterial efficacy of the extracts was assessed using the agar well diffusion method. Mueller-Hinton Agar plates were first prepared and uniformly inoculated with the respective test bacteria, *S. aureus* or *S. typhi*. Wells were then created in the inoculated agar using a sterile 7 mm diameter cork borer. These wells were subsequently filled with 50 µL of *I. longiflora* leaf extract at three distinct test concentrations: 40%, 60%, and 80% (v/v). For comparative analysis, 100% DMSO was used as the negative control. The positive controls consisted of standardized antibiotic discs: Ampicillin (10 µg) for *S. aureus* and Chloramphenicol (30 µg) for *S. typhi*. The prepared Petri dishes were then incubated at 37°C for 24 hours. Following the incubation period, the resulting diameter of the zone of inhibition around each well and antibiotic disc was measured to quantify the antibacterial activity[10].

Phytochemical screening test

Alkaloid test: A 0.5 g portion of *I. longiflora* leaf extract was combined with 1 mL of 2 N HCl and 9 mL of distilled water in a test tube. This mixture was heated for 2 minutes, cooled to room temperature, and subsequently filtered. The resulting filtrate was then treated with two drops of Dragendorff reagent. The formation of an orange precipitate confirmed the presence of alkaloids[11].

Flavonoid test: As much as 0.5 g of *I. longiflora* leaf extract was placed in a test tube and mixed with 5 mL of distilled water. This suspension was heated for 5 minutes and then filtered. To the collected filtrate, 0.1 g of magnesium powder and 1 mL of concentrated HCl were added, followed by thorough homogenization. A positive result for flavonoids was established by the distinct development of a yellow, orange, or red color[11].

Saponin test: As much as 0.5 g of *I. longiflora* leaf extract was added to a test tube containing 10 mL of distilled water and was then vigorously shaken. The presence of saponins was indicated by the formation of stable foam that persisted for a minimum of 10 minutes, with a foam height ranging from 1 to 10 cm. Crucially, the foam was required to remain stable and not dissipate upon the subsequent addition of 1 mL of 2 N HCl[11].

Phenolic test: For the phenolic assay, 0.5 g of *I. longiflora* leaf extract was transferred to a test tube, followed by the addition of 1 mL of 10% $FeCl_3$ solution. A positive result for phenolic compounds was characterized by the instantaneous appearance of a dark blue, blue-black, or greenish-black color[11].

Thin-layer chromatography analysis

The phytochemical constituents present in the 85% and 96% ethanol extracts of *I. longiflora* leaves were separated and analyzed using the Thin-layer chromatography (TLC) method. The stationary phase employed was a pre-coated silica gel GF_{254} plate. Plates were first activated by heating them at 100°C for 30 minutes to ensure optimal adsorbent performance[12].

The separation chamber was then prepared by saturating it with the mobile phase, a solvent system consisting of *n*-hexane : ethyl acetate (5 : 5), using filter paper. Before use, the TLC plate was precisely marked: the baseline (application point) was marked 1 cm from the bottom edge, and the solvent front (elution endpoint) was marked 0.5 cm from the top edge, establishing a total elution distance of 5 cm. Following the sample application at the baseline and allowing it to dry, the plate was eluted until the solvent reached the marked front. The separated spots were initially visualized under UV light at wavelengths of 254 nm and 366 nm. Subsequent phytochemical identification was performed by spraying the plate with specific reagents: Dragendorff's reagent for alkaloids, cytroborate reagent for flavonoids, and 10% $FeCl_3$ solution for phenolic compounds[13]. Observation and documentation of the results involved noting the number and color of the spots and calculating the retardation factor (Rf) for each separated compound using the following standard **Equation 2**[14]:

$$Rf = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by solvent}} \quad [2]$$

Contact bioautography assay

Contact bioautography was employed to identify the specific active compound groups within *I. longiflora* leaf 96% ethanol extract is responsible for the observed antibacterial activity[15]. First, the TLC plate, which had been previously eluted, was gently and firmly placed, using sterile tweezers, onto the surface of solid MHA media that had been pre-inoculated with *S. aureus*. The plate was left in direct contact with the inoculated agar for 1 hour to allow for the diffusion of active compounds. Subsequently, the chromatogram was carefully lifted from the media, and the MHA plate was incubated in an inverted position at 37°C for 24 hours. Following incubation, the diameter of any resulting inhibition zone was measured. The Rf of the inhibition zone was calculated and directly compared to the Rf values of the spots observed on the original TLC plate. Compounds possessing antibacterial efficacy were identified by matching the Rf value of the clear zone of inhibition to the corresponding Rf value of a chemical spot on the chromatogram.

Data analysis

The data acquired from the antibacterial activity test were subjected to statistical analysis utilizing SPSS version 25. Initial prerequisite analyses included checking the data for assumptions of normality and homogeneity of variances, both conducted at a 95% confidence level ($\alpha = 0.05$). Since the data typically did not meet the stringent parametric assumptions for both normality and homogeneity, the analysis was continued using the Kruskal-Wallis non-parametric statistical test to determine if significant differences existed among the treatment groups.

RESULTS AND DISCUSSION

Extraction, defined as the process of isolating target compounds from a complex mixture using an appropriate solvent, was optimized using UAE, a method that enhances mass transfer through the mechanical effects of ultrasonic waves. In this study, the efficiency of ethanol as an extraction solvent was evaluated at concentrations of 70%, 85%, and 96%. The resulting concentrated extracts of *I. longiflora* leaves were quantified, and the percentage extract yield is comprehensively presented in **Table I**. The highest yield of *I. longiflora* leaf extract was consistently achieved using a 70% ethanol solvent, compared to the higher concentrations of 85% and 96%. This observation aligns with the principle that increasing the ethanol concentration decreases the overall polarity of the hydroethanolic mixture, potentially reducing the solubility of the predominant compounds in the *I. longiflora* matrix at higher ethanol percentages[16].

Furthermore, the UAE method proved to be markedly more efficient than conventional maceration. Prior studies investigating *I. longiflora* maceration reported substantially lower yields despite using larger amounts of starting material and solvent over longer durations: Sunnah *et al.*[17] achieved a 5.092% yield from 500 g of *I. longiflora* leaf simplicia using 5,000 mL of 96% ethanol over 5 days, and Awwaliyah *et al.*[18] obtained a 17.64% yield from 300 g of simplicia using 3,000 mL of 70% ethanol over 3 days. Comparatively, the successful application of UAE demonstrates a significant advantage, producing a greater extract yield while requiring a smaller quantity of dry simplicia, a reduced solvent volume, and a substantially shorter extraction time than the traditional maceration technique[19].

Table I. Yield results of *I. longiflora* leaf thick extracts obtained by UAE.

Extract	Color	Initial weight of dried simplisia (g)	Weight of thick extract (g)	Yield (%)
Ethanol 70%	Dark brown	50.00	9.03	18.06
Ethanol 85%	Dark brown	50.00	7.29	14.58
Ethanol 96%	Dark brown	50.00	7.16	14.32

To establish appropriate positive controls for the subsequent antibacterial assays, bacterial sensitivity tests were conducted to evaluate the efficacy of various commercial antimicrobial agents against the test strains, quantified by measuring the diameter of the inhibition zone[20]. The antibiotic yielding the largest zone of inhibition against a specific bacterium is traditionally designated as the positive control. As detailed in **Table II**, the sensitivity testing revealed distinct optimal agents for the two target species. For *S. aureus*, the antibiotic that produced the highest inhibition zone diameter was Ampicillin (10 µg), which was therefore selected as the positive control. Conversely, against *S. typhi*, Chloramphenicol (30 µg) elicited the maximum inhibition response and was thus chosen as the positive control for the assays targeting this Gram-negative pathogen. These selected positive controls serve as benchmarks to ensure the validity and efficacy of the antibacterial tests.

Table II. Antibiotic sensitivity test results.

Bacteria	Antibiotic (disc concentration; µg)	Inhibition zone diameter (mm)
Staphylococcus aureus	Ampicillin (10)	38
	Chloramphenicol (30)	28
	Tetracycline (30)	30
	Vancomycin (30)	18
Salmonella typhi	Ampicillin (10)	24
	Chloramphenicol (30)	26
	Erythromycin (15)	10
	Vancomycin (30)	6

Note: Diameter including disc diameter of 6 mm

The antimicrobial potential of the *I. longiflora* leaf extracts was evaluated using the well diffusion method, with the resulting inhibition zone diameters serving as the key measure of efficacy[21]. To prepare the samples, a serial dilution of the leaf extract was prepared by dissolving it in 100% DMSO. DMSO served as the negative control to rigorously exclude any potential antimicrobial bias stemming from the solvent itself. For the positive controls, Ampicillin was used against *S. aureus*, and Chloramphenicol was used against *S. typhi*. The antibacterial activity was tested at three extract concentrations: 40%, 60%, and 80%. All tests were conducted in triplicate to ensure the reliability and accuracy of the measurements. The detailed results for the inhibition zone diameters are summarized in **Table III**.

Table III. Inhibition zone diameter of *I. longiflora* leaf ethanol extracts.

Extract	Inhibition zone diameter ± SD (mm)				
	Extract concentration (%)			Positive control	Negative control
	40	60	80		
Staphylococcus aureus				**Ampicillin**	**DMSO**
Ethanol 70%	9.2 ± 0.8	11.3 ± 0.6	11.7 ± 0.6	38.0 ± 0	7.0 ± 0
Ethanol 85%	7.2 ± 0.3	7.3 ± 0.3	7.5 ± 0.5	38.0 ± 0	7.0 ± 0
Ethanol 96%	12.2 ± 0.6	12.8 ± 0.3	13.7 ± 1.2	38.0 ± 0	7.0 ± 0
Salmonella typhi				**Chloramphenicol**	**DMSO**
Ethanol 70%	7.0 ± 0	7.0 ± 0	7.0 ± 0	24.7 ± 1.2	7.0 ± 0
Ethanol 85%	8.8 ± 0.8	9.3 ± 1.2	9.5 ± 0.9	24.7 ± 1.2	7.0 ± 0
Ethanol 96%	7.0 ± 0	7.0 ± 0	7.0 ± 0	24.7 ± 1.2	7.0 ± 0

The most potent inhibitory effect against *S. aureus* was observed with the 96% ethanol extract prepared using the UAE method. At the highest tested concentration of 80%, this extract yielded an average inhibition zone diameter of 13.7 mm. This finding is strongly supported by an earlier study by Angganawati and Nisa[7], which tested a 96% ethanol extract of *I. longiflora* leaves obtained through maceration. That study reported a maximal inhibition zone diameter of 14.3 mm against *S. aureus* at a concentration of 300 mg/mL, demonstrating comparable and robust activity from 96% ethanol extracts regardless of the extraction method (UAE vs. maceration). Conversely, against *S. typhi*, the 85% ethanol extract demonstrated the highest inhibitory zone diameter. This suggests that the metabolite compounds responsible for inhibiting *S. typhi* were more efficiently and selectively extracted by the 85% ethanol solvent compared to the 96% or other

concentrations tested. Crucially, a concentration-dependent effect was consistently observed across all tested extracts; as the extract concentration increased, a corresponding and proportional increase in the diameter of the inhibition zone was noted. This result is consistent with the findings of Wardani *et al.*[4], which also established that increasing the concentration of *I. longiflora* leaf extract leads to a significantly greater diameter of inhibition.

The inhibition zone diameter data for both tested bacterial species were subjected to statistical analysis using SPSS version 25. Initial assessment revealed that the data violated the fundamental assumptions of normality and homogeneity of variances. Consequently, the one-way ANOVA test was deemed inappropriate, necessitating the use of the non-parametric Kruskal-Wallis test instead. The results from the Kruskal-Wallis test indicated a statistically significant difference in the diameter of the inhibition zone between the 85% ethanol extract and the 96% ethanol extract when tested against *S. aureus*. Furthermore, a notable significant difference was observed in the inhibition zone diameter against *S. typhi* across the different solvent concentrations, specifically comparing the 85% ethanol extract with both the 70% ethanol extract and the 96% ethanol extract. Interestingly, despite these significant solvent-dependent differences, variations in extract concentration within each solvent type did not result in a statistically significant difference in the resulting inhibition zone diameter for either of the test bacteria.

Phytochemical screening was subsequently performed on the specific extracts that demonstrated the highest inhibition zone diameter against each respective test bacterium: the 85% and 96% ethanol extracts. The general methodology involved placing a small amount of the extract in a test tube and adding the appropriate detection reagent; an observable color change or precipitate formation served as an indicator of the presence of a specific compound class[22]. **Table IV** summarizes the findings, confirming that both the 85% and 96% ethanol extracts of *I. longiflora* leaves contained alkaloids, flavonoids, saponins, and phenolics.

Table IV. Qualitative phytochemical screening for 85% and 96% ethanol extracts of *I. longiflora* leaves.

Compound class	85% Ethanol extract result	96% Ethanol extract result
Alkaloids	(+) Orange-red precipitate (with Dragendorff reagent)	(+) Orange precipitate (with Dragendorff reagent)
Flavonoids	(+) Solution turned red-brown	(+) Solution turned orange
Saponins	(+) Stable foam with a height of 1.5 cm	(+) Stable foam with a height of 2.5 cm
Phenolics	(+) Blackish green color	(+) Blackish green color

The presence of alkaloids was verified by the formation of a precipitate upon the addition of Dragendorff reagent. This precipitate is a complex of potassium tetraiodobismutate, which forms through the reaction between bismuth nitrate and potassium iodide, ultimately yielding bismuth (III) iodide soluble in potassium iodide[11]. For the flavonoid test, the use of concentrated HCl reagent and magnesium powder is a reduction reaction. These reagents facilitate the reduction of the benzopyrone core characteristic of the flavonoid structure, resulting in the formation of colored flavylium salts that manifest as a distinct red or orange color[23]. The positive test for saponins involves the formation of persistent foam upon shaking. Saponins are surfactants containing both hydrophilic and hydrophobic groups. The interaction of the hydrophilic groups with water and the hydrophobic groups with air during agitation stabilizes the foam. The deliberate addition of 2N HCl in this protocol is intended to increase the polarity, which reinforces the bonds between the hydrophilic groups, thereby ensuring the stability of the foam[11]. Finally, the presence of phenolic compounds was confirmed by the observation of a blackish-green color following the addition of 10% $FeCl_3$. This color change results from the formation of complex compounds between the phenolic molecules (such as tannins) and the Fe^{3+} ions, with a positive result classically indicated by the emergence of a dark blue, blue-black, or blackish-green hue[24].

The TLC analysis was executed to definitively identify and verify the presence of targeted secondary metabolite groups within the extracts. The chromatographic system employed a silica gel GF_{254} plate as the stationary phase and a mixture of *n*-hexane : ethyl acetate (5 : 5) as the mobile phase. Specific spray reagents were utilized post-separation to visualize and characterize the compound spots. Identification focused on alkaloids, flavonoids, and phenolic compounds, using Dragendorff's reagent, $AlCl_3$ citroborate reagent, and 10% $FeCl_3$ solution, respectively. The resulting chromatograms for the 85% ethanol extract and the 96% ethanol extract of *I. longiflora* leaves, both prepared using the UAE method, are detailed in **Figures 1** and **2**. Upon spraying with Dragendorff's reagent, an orange-brown spot was observed under visible light, confirming the presence of alkaloid compounds. The detection of flavonoids was achieved using the citroborate reagent, which yielded a characteristic yellow spot when viewed under UV light at 366 nm. Furthermore, the presence of phenolic

compounds was evidenced by the appearance of a dark green or blackish spot under visible light following application of the 10% $FeCl_3$ reagent. Collectively, the TLC results confirmed that both the 85% and 96% ethanol extracts of *I. longiflora* leaves, extracted via UAE, contain alkaloid, flavonoid, and phenolic compounds.

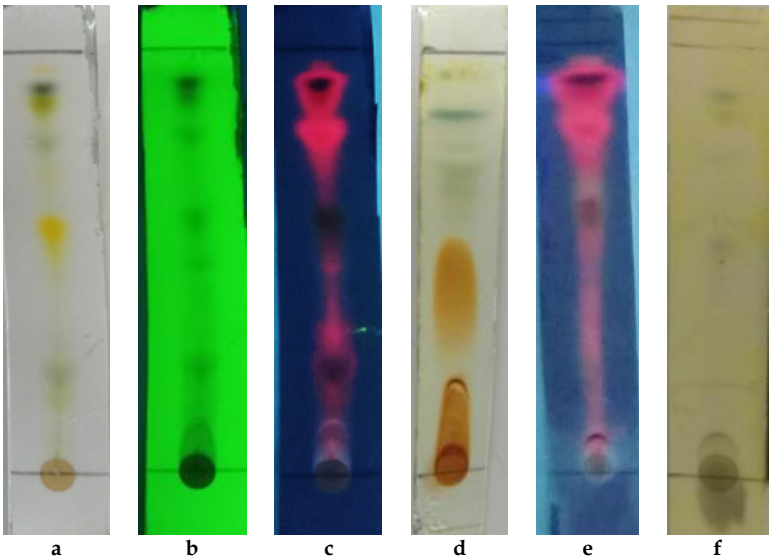

Figure 1. TLC results of the 85% ethanol extract of *I. longiflora* leaves (UAE). Visualization methods include: (a) Visible light, (b) UV 366 nm, (c) UV 254 nm, (d) Alkaloid detection using Dragendorff reagent (Visible light), (e) Flavonoid detection using cytroborate reagent (UV 366 nm), and (f) Phenolic detection using $FeCl_3$ reagent (Visible light).

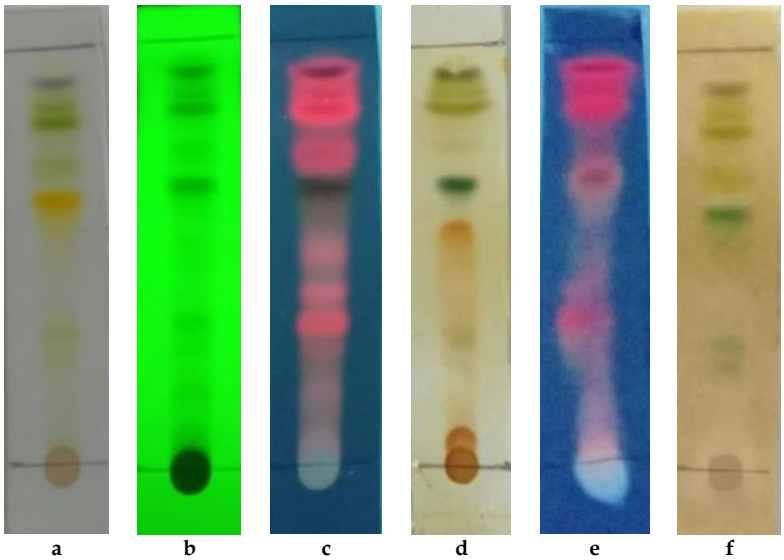

Figure 2. TLC results of the 96% ethanol extract of *I. longiflora* leaves (UAE). Visualization methods include: (a) Visible light, (b) UV 366 nm, (c) UV 254 nm, (d) Alkaloid detection using Dragendorff reagent (Visible light), (e) Flavonoid detection using cytroborate reagent (UV 366 nm), and (f) Phenolic detection using $FeCl_3$ reagent (Visible light).

The objective of the TLC-bioautography assay was to qualitatively identify the antimicrobial metabolite compounds present in the 96% ethanol extract of *I. longiflora* leaves against *S. aureus* using the contact bioautography method. A known limitation of this specific technique is the difficulty in ensuring optimal and consistent contact between the agar medium and the chromatogram plate, which can result in the transfer of bacterial suspension upon separation[25]. The extract was tested in triplicate, alongside a control plate, to compare the presence of clear zones indicative of inhibition.

The results, as depicted in **Figure 3**, showed that no clear zone of inhibition was observed on the extract plates, indicating no detectable antimicrobial activity via this method. Furthermore, there was no discernible difference between the extract plates and the control plates. This absence of localized inhibition suggests a potential challenge in isolating an individual active compound via TLC that possesses a potent, singular inhibitory effect. One hypothesis for this result relates to a

synergistic or additive effect among the compounds in the crude ethanol extract. It is plausible that while the entire extract may exhibit antimicrobial properties (as often assessed in dilution methods), the individual, separated components lack sufficient potency alone, necessitating the combined action of multiple compounds to exert an inhibitory impact on the test bacteria[26].

Figure 3. TLC-bioautography profile of (left) control and (right) *I. longiflora* ethanol leaf extract against *S. aureus*.

Phytochemical screening of the *I. longiflora* ethanol leaf extract confirmed the presence of alkaloids, flavonoids, saponins, and phenolic compounds. This composition is consistent with prior research conducted by Eff[5], which also reported the presence of alkaloids, flavonoids, saponins, and tannins in the same extract. These identified compound classes possess well-established mechanisms of antibacterial action. Alkaloids typically inhibit bacterial growth by interfering with the synthesis or integrity of peptidoglycan, leading to defective cell wall formation and subsequent cell death. Flavonoid compounds exert their effect by forming complex compounds with extracellular and soluble proteins, which ultimately disrupts the bacterial cell membrane integrity. Saponins reduce the cell surface tension, increasing membrane permeability and causing cellular leakage and the release of essential intracellular components[27]. Finally, phenolic compounds employ multiple mechanisms, including direct damage to the bacterial membrane, inhibition of virulence factors, and suppression of bacterial biofilm formation[28]. The presence of these combined bioactive components underscores the potential of *I. longiflora* as a natural antimicrobial source, despite the negative findings from the TLC-bioautography assay, which primarily reflects the activity of isolated elements.

CONCLUSION

In summary, the antibacterial efficacy of *I. longiflora* leaf extract exhibited solvent-dependent selectivity. Specifically, the extract prepared using 96% ethanol via UAE demonstrated the most potent activity against *S. aureus*, yielding the largest inhibition zone diameter. Conversely, the extract employing 85% ethanol provided the superior inhibition zone against *S. typhi*. Phytochemical analysis, conducted via tube tests and TLC, confirmed the presence of key secondary metabolites in the ethanol extract, including alkaloids, flavonoids, saponins, and phenolic compounds.

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AUTHORS' CONTRIBUTION

Conceptualization: Ika Trisharyanti Dian Kusumowati
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Resources: Fajrin Ahidannisa Yuhdi
Software: -
Supervision: Ika Trisharyanti Dian Kusumowati
Validation: Ika Trisharyanti Dian Kusumowati
Visualization: Fajrin Ahidannisa Yuhdi
Writing - original draft: Fajrin Ahidannisa Yuhdi
Writing - review & editing: Ika Trisharyanti Dian Kusumowati

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declared no conflict of interest related to this research.

REFERENCES

1. Permana A, Aulia SD, Azizah NN, Rudhiana T, Suci SE, Izzah INL, et al. Artikel Review : Fitokimia dan Farmakologi Tumbuhan Kitolod (Isotoma longiflora Presi). J Buana Farma. 2022;2(3):22-35. DOI: 10.36805/jbf.v2i3.547

2. Nazir A, Nazir A, Zuhair V, Aman S, Sadiq SUR, Hasan AH, et al. The Global Challenge of Antimicrobial Resistance: Mechanisms, Case Studies, and Mitigation Approaches. Health Sci Rep. 2025;8(7):e71077. DOI: 10.1002/hsr2.71077; PMCID: PMC12284435; PMID: 40704322

3. Kumar P, Kumar D, Gautam V, Singh S, Pratama MRF. Antimicrobial Resistance a Global Burden - Mechanisms, Current Insights, and Future Directions. Bull Pharm Sci Assiut Univ. 2025;48(1):523-45. DOI: 10.21608/bfsa.2024.310197.2228

4. Wardani TS, Nisa TC, Artini KS. Antibacterial Activity Test of N-Hexan, Ethyl Acetate and Water from Ethanol Extract of Kitolod Leaf (Isotoma Longiflora (L.) C. Presl.) AGAINST Staphylococcus Aureus ATCC 25923. Proceed Int Conf Nurs Health Sci. 2022;3(1):9-16. DOI: 10.37287/picnhs.v3i1.984

5. Eff ARY. Uji Sitotoksik Ekstrak Etanol 50% Daun Kitolod (Isotoma longiflora (L.) Presl.) terhadap Sel Kanker Serviks (Ca Ski Cell Line) secara In- Vitro. Farmasains J Ilmiah Ilmu Kefarmasian. 2016;3(1):7-12.

6. Anjelina SH. Antibacterial Activity of Ethanolic Extract of Kitolod (Hippobromalongiflora) Leaf Against Staphylococcus aureus and Salmonella typhi. Asian J Pharm Res Dev. 2020;8(1):52–4. DOI: 10.22270/ajprd.v8i1.660

7. Angganawati RT, Nisa TC. Uji Aktivitas Antibakteri Ekstrak Etanol Daun Kitolod (Isotoma longiflora (L.) C. Presl.) terhadap bakteri Staphylococcus aureus dengan Kontrol Antibiotik Ofloxacin. J Farmasindo J Penelitian Pengabdian Masyarakat. 2019;3(1):5–8. DOI: 10.46808/farmasindo.v3i1.14

8. Shen L, Pang S, Zhong M, Sun Y, Qayum A, Liu Y, et al. A comprehensive review of ultrasonic assisted extraction (UAE) for bioactive components: Principles, advantages, equipment, and combined technologies. Ultrason Sonochem. 2023;101:106646. DOI: 10.1016/j.ultsonch.2023.106646; PMCID: PMC10594638; PMID: 37862945

9. Mubarak F, Sartini S, Purnawanti D. Effect of Ethanol Concentration on Antibacterial Activity of Bligo Fruit Extract (Benincasa hispida Thunb) to Salmonella typhi. Indones J Pharm Sci Technol. 2018;5(3):76-81. DOI: 10.24198/ijpst.v5i3.16444

10. Phuyal N, Jha PK, Raturi PP, Rajbhandary S. In Vitro Antibacterial Activities of Methanolic Extracts of Fruits, Seeds, and Bark of Zanthoxylum armatum DC. J Trop Med. 2020;2020:2803063. DOI: 10.1155/2020/2803063; PMCID: PMC7292994; PMID: 32565829

11. Maheshwaran L, Nadarajah L, Senadeera SPNN, Ranaweera CB, Chandana AK, Pathirana RN. Phytochemical Testing Methodologies and Principles for Preliminary Screening/ Qualitative Testing. Asian Plant Res J. 2024;12(5):11-38. DOI: 10.9734/aprj/2024/v12i5267

12. Kowalska T, Sajewicz M. Thin-Layer Chromatography (TLC) in the Screening of Botanicals-Its Versatile Potential and Selected Applications. Molecules. 2022;27(19):6607. DOI: 10.3390/molecules27196607; PMCID: PMC9572063; PMID: 36235143

13. Kancherla N, Dhakshinamoothi A, Chitra K, Komaram RB. Preliminary Analysis of Phytoconstituents and Evaluation of Anthelminthic Property of Cayratia auriculata (In Vitro). Maedica. 2019;14(4):350-6. DOI: 10.26574/maedica.2019.14.4.350; PMCID: PMC7035446; PMID: 32153665

14. Spangenberg B, Poole CF, Weins C. Quantitative Thin-Layer Chromatography: A Practical Survey. Heidelberg: Springer Berlin; 2011. DOI: 10.1007/978-3-642-10729-0

15. Tock MLA, Combrinck S, Kamatou G, Chen W, Vuuren SV, Viljoen A. Antibacterial Screening, Biochemometric and Bioautographic Evaluation of the Non-Volatile Bioactive Components of Three Indigenous South African Salvia Species. Antibiotics. 2022;11(7):901. DOI: 10.3390/antibiotics11070901; PMCID: PMC9312202; PMID: 35884155

16. Armonavičius D, Maruška A, Jakštys B, Stankevičius M, Drevinskas T, Bimbiraitė-Survilienė K, et al. Evaluation of the Anticancer Activity of Medicinal Plants Predominantly Accumulating Ellagic Acid Compounds. Antioxidants. 2025;14(11):1339. DOI: 10.3390/antiox14111339; PMCID: PMC12649554; PMID: 41300496

17. Sunnah I, Dianingati RS, Wulandari AR. Optimasi Pelarut terhadap Parameter Spesifik Ekstrak Kitolod (Isotoma longiflora). Generics J Res Pharm. 2021;1(1):10–5. DOI: 10.14710/genres.v1i1.9847

18. Awwaliyah R, Muslikh FA, Abada I, Megawati DS, Inayatillah R, Wijaya D, et al. Aktivitas Penyembuhan Luka Formulasi Salep Ekstrak Etanol Daun Kitolod (Isotoma longiflora) pada Mencit (Mus musculus). J Dunia Farmasi. 2023;7(2):105-15. DOI: 10.33085/jdf.v7i2.5425

19. Ariestanti DM, Mun'im A, Hartrianti P, Nadia B, Chriscensia E, Rattu SA, et al. Ultrasonic-assisted extraction (UAE) of Javanese turmeric rhizomes using natural deep eutectic solvents (NADES): Screening, optimization, and in vitro cytotoxicity evaluation. Ultrason Sonochem. 2025;114:107271. DOI: 10.1016/j.ultsonch.2025.107271; PMCID: PMC11872070; PMID: 39955874

20. Hossain TJ. Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. Eur J Microbiol Immunol. 2024;14(2):97-115. DOI: 10.1556/1886.2024.00035; PMCID: PMC11097785; PMID: 38648108

21. Nurafifah OA, Lantang D, Pratiwi RD, Dewi K, Bakri NF. Antibacterial Activity of Kitolod (Isotoma Longiflora L.) Herb Extract and Fractions Against the Growth of Dental Carises Causing Bacteria Streptococcus Mutans. Int J Pharm Bio Med Sci. 2025;5(4):227-32. DOI: 10.47191/ijpbms/v5-i4-02

22. Ahmad R, Amiruddin R, Arsin A, Stang S, Ishak H, Wahiduddin, et al. Phytochemical Screening and Antibacterial Activity Test of Ethanol Extract of Durian (Durio Zibethinus murr.) Soya Varieties Against Pathogen Bacteria Escherichia Coli in Raw Drinking Water. Pharmacogn J. 2024;16(4):933-41. DOI: 10.5530/pj.2024.16.151

23. Kefayati Z, Motamed SM, Shojaii A, Noori M, Ghods R. Antioxidant Activity and Phenolic and Flavonoid Contents of the Extract and Subfractions of Euphorbia splendida Mobayen. Pharmacogn Res. 2017;9(4):362-5. DOI: 10.4103/pr.pr_12_17; PMCID: PMC5717788; PMID: 29263629

24. Godlewska K, Pacyga P, Najda A, Michalak I. Investigation of Chemical Constituents and Antioxidant Activity of Biologically Active Plant-Derived Natural Products. Molecules. 2023;28(14):5572. DOI: 10.3390/molecules28145572; PMCID: PMC10384900; PMID: 37513443

25. Wang M, Zhang Y, Wang R, Wang Z, Yang B, Kuang H. An Evolving Technology That Integrates Classical Methods with Continuous Technological Developments: Thin-Layer Chromatography Bioautography. Molecules. 2021;26(15):4647. DOI: 10.3390/molecules26154647; PMCID: PMC8347725; PMID: 34361800

26. Urma SRA, Ahmed SF, Imran MAS, Akhand MRN, Khan MMH. Antimicrobial Activity of Tea and Agarwood Leaf Extracts Against Multidrug-Resistant Microbes. Biomed Res Int. 2024;2024:5595575. DOI: 10.1155/bmri/5595575; PMCID: PMC11671646; PMID: 39734496

27. Zhang Z, Cao M, Shang Z, Xu J, Chen X, Zhu Z, et al. Research Progress on the Antibacterial Activity of Natural Flavonoids. Antibiotics. 2025;14(4):334. DOI: 10.3390/antibiotics14040334; PMCID: PMC12023951; PMID: 40298463

28. Buchmann D, Schwabe M, Weiss R, Kuss AW, Schaufler K, Schlüter R, et al. Natural phenolic compounds as biofilm inhibitors of multidrug-resistant Escherichia coli - the role of similar biological processes despite structural diversity. Front Microbiol. 2023;14:1232039. DOI: 10.3389/fmicb.2023.1232039; PMCID: PMC10507321; PMID: 37731930