



Antimicrobial Activity of Khyung Inga, a Bhutanese Traditional Medicine, Against *Staphylococcus aureus*: An In Vitro Study

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Received: December 1, 2025 / **Revised:** February 15, 2026 / **Accepted:** February 24, 2026 / **Published:** April 8, 2026

Abstract

Background: Khyung Inga, a Bhutanese traditional medicine, is composed of five crude ingredients and is commonly used to treat skin diseases, leprosy, neck stiffness, rheumatic arthritis, abscesses and stomach cramps in Sowa Rigpa, and is registered with Bhutan's Drug Regulatory Authority. Despite its widespread use, no scientific evidence supports its antimicrobial properties. To address this gap, two batches of crude Khyung Inga extract, prepared in 2016 and 2018, were tested in 2019 for antimicrobial activity against four bacteria.

Methods: The sample extraction was performed using the cold extraction method, as outlined by Addo PW, which is currently used by Menjong Sorig and Pharmaceutical Corporation Limited to extract small samples. To test the antimicrobial properties, the Kirby-Bauer disc diffusion method was used, following guidelines from the Royal Center for Disease Control, Ministry of Health, Bhutan.

Results: The Khyung Inga batch manufactured in 2018 demonstrated a maximum inhibition zone of 20 mm at a concentration of 1000 mg/10 ml. The 2016 batch of Khyung Inga showed slightly lower inhibition compared to the 2018 batch. Two-factor analysis of variance revealed significant differences between batches ($P < 0.05$) and among concentrations ($P < 0.01$), with higher inhibition zones observed for the 2018 batch and at higher concentrations.

Conclusion: Recent studies show that Khyung Inga has antimicrobial activity against *Staphylococcus aureus*, a bacterium responsible for skin infections, such as abscesses and boils. This suggests it can be used as a complementary treatment, reducing reliance on imported medicines. However, further in vivo studies are needed to confirm its clinical effectiveness.

Keywords: Antimicrobial agents, Disc diffusion, Sowa Rigpa, *Terminalia chebula*, Traditional medicine



Introduction

Bhutanese Traditional Medicine Services currently produces approximately 113 traditional medicine formulations. Among these, nine medicines are specifically prepared to treat infections known as "srin men." Khyung lnga, one of these nine srin mens, is widely used across the country for various infections. It effectively addresses skin conditions, such as leprosy, neck stiffness and inflammation, rheumatic arthritis, abscesses, and stomach cramps. This formulation draws from Bhutanese traditional medicine (Sowa Rigpa), which integrates over 100 recognized preparations tailored to local needs, emphasizing anti-inflammatory and antimicrobial properties (1).

Khyung lnga (KH) is prepared from five crude ingredients (*Terminalia chebula* Retzius, *Aconitum laciniatum*, *Acorus calamus* L., *Saussurea lappa*, and *Delphinium Brunonianum*) and follows standard manufacturing procedures outlined in the Sowa Rigpa text. It comes in dark black 50 mg pills with a distinctive odor and a hot intrinsic nature (2). These medicines have been used for many years to treat infections and are registered with the Drug Regulatory Authority (DRA) of Bhutan as essential drugs in the traditional medicine healthcare system; however, no scientific literature states that they have antimicrobial activity (3).

Therefore, we screened two batches of crude Khyung lnga extracts manufactured in 2016 and 2018 for antimicrobial activity against four bacteria in 2019, to validate the classical Sowa Rigpa text against evidence-based literature.

Materials and Methods

Test Medicine

Two batches of KH, which were manufactured in 2016 and 2018, were collected from the National Traditional Medicine Hospital. During analysis, the samples were recoded as KH-I for the 2016 batch and KH-II for the 2018 batch.

KH is compounded from five key ingredients. Two of them, A-ru (*Terminalia chebula* Retzius) and Shu-dag nagpo (*Acorus calamus* L.), are low-altitude medicinal plants collected from southern regions of Bhutan, such as Trongsa and Gelephu,

which are below 3,000 meters above sea level. The other three ingredients, Bong-nag (*Aconitum laciniatum*), Ru-rta (*Saussurea lappa*), and Byad-rgod-spos (*Delphinium brunonianum*), are high-altitude medicinal plants sourced from elevations of 3,000 to 6,000 meters in northern Bhutan, including Lingzhi, Bumthang, and Haa regions.

Study Site

The antimicrobial activity of the proposed traditional medicines was primarily evaluated at the Royal Center for Disease Control (RCDC) in Wangchuk Taba, Thimphu, Bhutan¹. Wangchuk Taba is situated approximately 16 kilometers southeast of Thimphu. RCDC serves as the nation's sole facility for generating reliable health and disease information through its robust public health and environmental surveillance systems, research initiatives, and state-of-the-art laboratory diagnostics.

The sample medicines were extracted at the Quality Control and Research Laboratory of Menjong Sorig Pharmaceuticals Corporation Limited (MSPCL) in Kawajangsa, Thimphu. MSPCL is the leading manufacturer and research and development center for traditional medicines, supplying these medicines to all Traditional Medicine units across the country.

Extract Preparation

The sample extraction followed the cold extraction method described by Addo PW, 2021 (4), which Menjong Sorig Pharmaceuticals Corporation Limited also uses for small-sample extractions. Initially, all laboratory equipment was thoroughly cleaned, dried, and prepared for use. Two batches of the proposed medicines, presented in pill form, were ground into coarse powder using a mortar and pestle. Exactly 50 grams of each powdered sample was weighed and soaked in absolute ethanol in an Erlenmeyer flask, maintaining a ratio of 1 gram to 3 milliliters of solvent (50 g: 150 mL). The mixture was occasionally stirred and left for 24 hours to ensure

¹ Nestled between India and China, Bhutan is a small nation with a population of approximately 790,000. Renowned worldwide as

the land of Gross National Happiness, the country boasts an integrated and universally free healthcare system.

complete extraction of the chemical constituents. The flask's mouth was sealed with aluminium foil during the process (Figure 1).

After 24 hours, the solution was filtered using a funnel and Whatman filter paper (110 mm). The medicine residue was rinsed twice with 20 mL of solvent each time to ensure complete extraction. Once filtration was complete, the solution was transferred to a 90 mm Petri dish for drying and evaporation. The Petri dish was weighed

beforehand, and the filtrate solution was evaporated and dried in a water bath at 75.5°C for 48 hours. The dried extract was then cooled for an additional 24 hours to achieve complete dryness. The extract was reweighed to calculate the total extraction percentage of all sample products, as presented in Table 1. Finally, the extract was stored in a refrigerator at 2°C to 8°C until analysis.

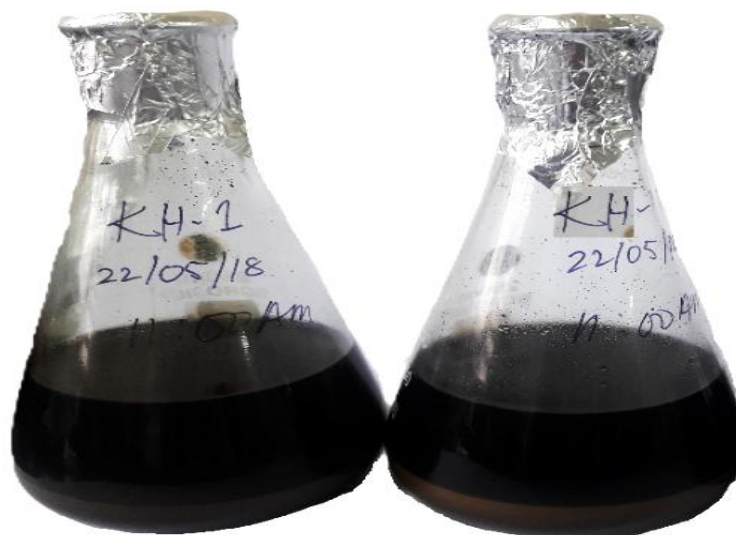


Figure 1. Cold Ethanol Extraction Process

Table 1. Total Yields of Crude Ethanol Extracts Obtained from Khyung lnga Batches KH-I (2016) and KH-II (2018) Using Cold Extraction Method (50 g Powder in 150 mL Ethanol)

Sample Medicines	Weight Powder	Volume of Solvent	Weight of Container	Extracted Weight with Container	Extract Obtained	Extract %
KH-I	50 g	150 mL	48.47	52.25	3.81	7.63
KH-II	50 g	150 mL	48.25	55.68	7.38	15.68

*khyung lnga batch I and II

The total extract percentage was calculated using the following formula:

Percentage of extract obtained=

$$\frac{\text{Extract obtained}}{50 \text{ g}} \times 100$$

Preparation of Sample Stock Solution and Two-Fold Serial Dilution

This method was used according to the American Association of Microbiology. A total of 1000 mg of proposed crude extract medicine was dissolved in 10 mL of double-distilled water to make a stock solution of 1000 mg/10 mL (w/v), or 100 mg/mL; then the mixture was mixed evenly and made homogeneous using a vortex mixer. The solution was stored at 2°C to 8°C when not in use.

Muller-Hinton agar was prepared as per the manufacturer's instructions (5).

The stock solution had a concentration of 1000 mg/10 mL. A series of dilutions was prepared by adding double-distilled water, as illustrated in Figure 1. First, 5 mL of distilled water was added to each test tube (B, C, and D). Then, 5 mL of the stock solution (A) was mixed thoroughly with 5 mL of distilled water in test tube B, creating a concentration of 500 mg/10 mL (50 mg/mL). The mixture was vortexed to ensure uniformity. Next, 5 mL of the solution from test tube B was

transferred to test tube C, mixed well, and diluted to a concentration of 250 mg/10 mL (25 mg/mL). Finally, 5 mL of the solution from test tube C was transferred to test tube D, mixed thoroughly to achieve a concentration of 125 mg/10 mL, and 0.5 mL was discarded after vortexing (5).

Test Organism

Test organisms were obtained from the Enteric Invasive Diseases Laboratory (EIDL) RCDC. Four organisms were used to test the medicine (Table 2).

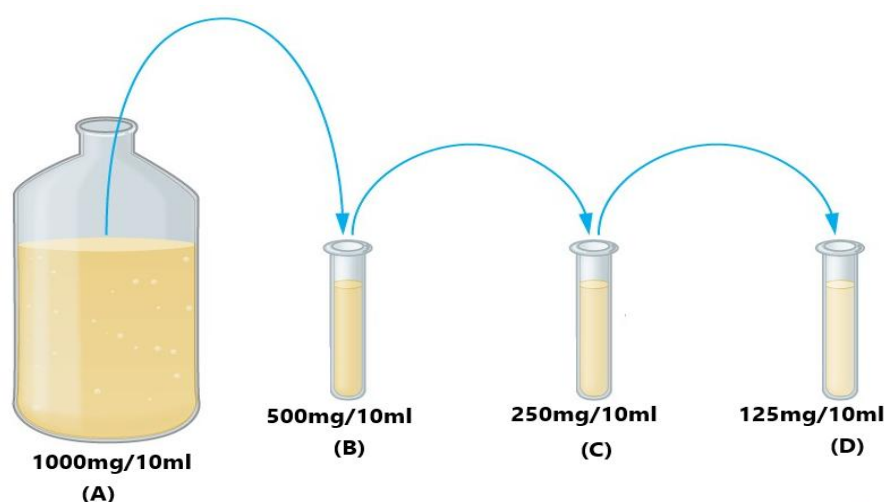


Figure 2. Two-Fold Serial Dilution Method

Table 2. Bacterial Strains Used in the Antimicrobial Susceptibility Testing of Khyung Inga Extracts

SI No.	Name of Organism	ATCC* No.
1	<i>Staphylococcus aureus</i> (<i>S.aureus</i>)	25923
2	<i>Escherichia coli</i> (<i>E-coli</i>)	29522
3	<i>Salmonella typhimurium</i> (<i>S. typhimurium</i>)	14028
4	<i>Pseudomonas aeruginosa</i> (<i>P.aeruginosa</i>)	27853

* American Type Culture Collection

SSub-culture of Test Micro-organisms

The materials required included Mueller-Hinton agar, 90 mm Petri dishes, a wire loop, a lamp, and an incubator. First, the Mueller-Hinton agar plate was dried in the incubator at 37°C for 30 minutes before inoculation. A sterilized wire loop was used to streak a small number of bacteria from the isolated ATCC test tube onto the plate. The

inoculated plate was then incubated at 37°C for 18-24 h (6).

Preparation of Bacterial Suspension

A bacterial suspension was prepared in normal saline (NaCl) to a standard concentration of 0.5 McFarland turbidity (7).

Preparation of Agar Plate

Mueller-Hinton agar (MHA) was used as the base medium for the study. The medium was prepared according to the manufacturer's instructions. Approximately 25 mL of melted agar was poured into the 90 mm Petri dish to obtain a thickness of 3-4 mm. The temperature of melted agar was maintained at 35- 45 °C. The agar plate was kept at room temperature for 30 minutes to dry it completely (8).

Antimicrobial Assays

Disc diffusion Method

The disc diffusion method was performed as per the Kirby-Bauer disc diffusion method following the guidelines of the Royal Center for Disease Control, Ministry of Health (9). The in vitro antimicrobial activity was tested using MHA obtained from the Royal Center for Disease Control in Thimphu. To evaluate the antibacterial properties of the sample medicines, the bacterial suspension was adjusted to a 0.5 McFarland standard. Then 100 µL of bacterial suspension was pipetted onto the surface of an MHA plate and spread evenly using a sterile swab stick under aseptic biosafety conditions. The plate was left to dry for 15 minutes.

Sterile discs impregnated with different concentrations of the sample medicines were placed on the surface of the swabbed MHA plate. A positive control, consisting of a commercial

standard disc of amoxicillin (10 µg), was included, and a negative control was left blank. All assays were performed in triplicate ($n = 3$ biological replicates per concentration), and mean inhibition zones $\pm$ SD were calculated using Stata 17. After incubation, the inhibition zones on the discs were measured in millimeters using a transparent ruler.

Impregnation of Disc

The sterile ready-made 6 mm disc was placed in the Petri dish using a mechanical pipette, and 20 µL of sample solution from a series of concentrations was loaded into the disc one by one. Without covering the Petri dish, the disc was allowed to dry in a clean incubator at 37°C for 4 hours (10).

Inoculation

The agar plate was dried for 10 to 15 minutes in a 37°C incubator prior to inoculation to maintain moisture. The plate was divided into different concentrations of the disc. A 100 µL bacterial suspension was pipetted onto the surface of the agar plate and spread evenly using a sterile swab stick under aseptic biosafety conditions. After that, the sampled medicine disc was placed in each division and incubated at 37°C for 24 hours (10). The assays were performed in triplicate ($n=3$ biological replicates per concentration), and the mean results were summarized (Table 3).

Table 3. Mean Zones of Inhibition (mm $\pm$ SD) Against Four Test Organisms from ($n = 3$ Biological Replicates per Concentration/Batch)

Organisms	<i>Staphylococcus aureus</i>				<i>Pseudomonas aeruginosa</i>				<i>Salmonella typhimurium</i>				<i>Escherichia coli</i>			
Concent/ml	1000	500	250	Amx	1000	500	250	Amx	1000	500	250	Amx	1000	500	250	Amx
*KH-I	18 $\pm$ 1	13 $\pm$ 1	7 $\pm$ 1	35	0	0	0	15	0	0	0	21	0	0	0	15
*KH-II	20 $\pm$ 1	15 $\pm$ 1	10 $\pm$ 1	35	7 $\pm$ 1	0	0	15	0	0	0	22	0	0	0	15

Two-factor analysis of variance revealed significant differences between batches ($P < 0.05$) and among concentrations ($P < 0.01$), with higher inhibition zones observed for the 2018 batch and at higher concentrations (Table 3). The effect of concentration was also statistically significant ($F = 198.6$, $P < 0.001$). This shows that antibacterial activity varied significantly across

the tested concentrations. Higher concentrations produced larger zones of inhibition compared to lower concentrations.

The interaction between batch and concentration was not statistically significant ($F=0.6$, $P=0.56$). This indicates that the effect of concentration on antibacterial activity was similar for both batches.

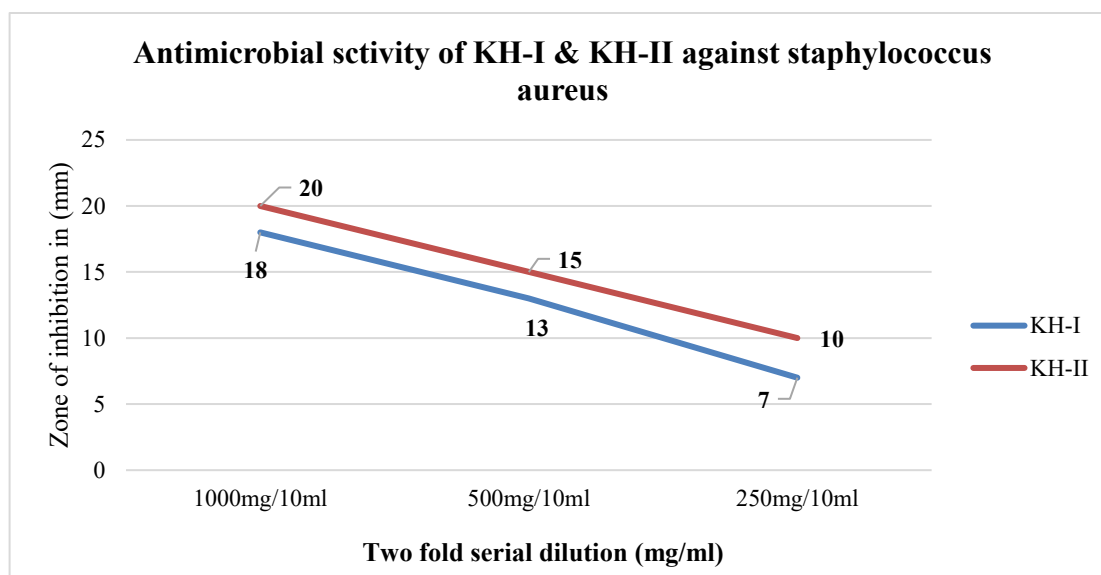


Figure 3. Mean Zones of Inhibition ($\pm$ SD) of Khyung Lnga Extracts (KH-I and KH-II) Against *Staphylococcus aureus* at Three Concentrations (1000, 500, 250 mg/10 mL) (n = 3 Biological Replicates per Concentration).

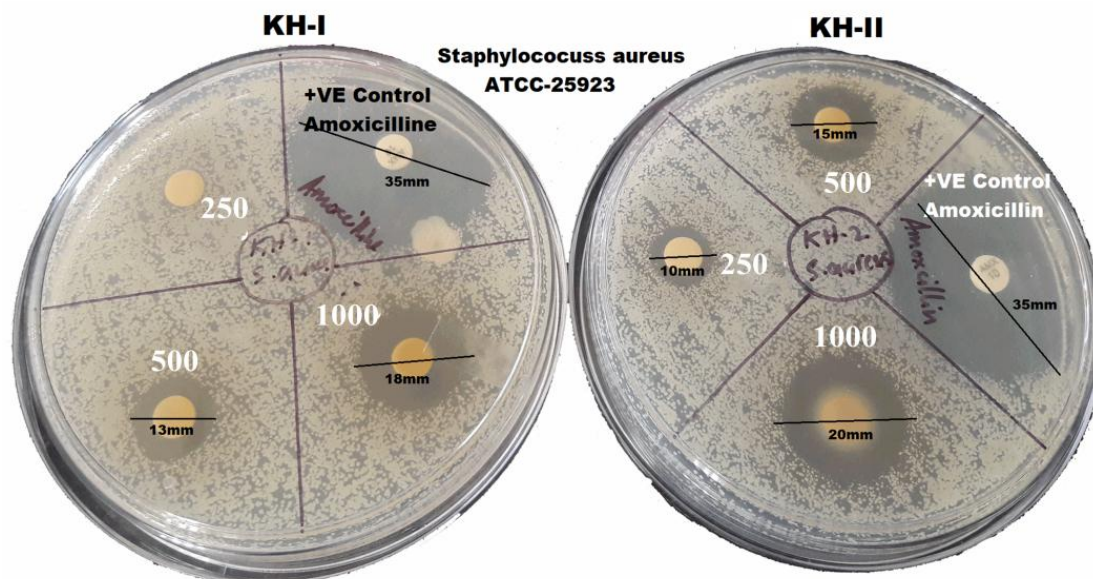


Figure 4. Visual Analysis of Zones of Inhibition among KH-I and KH-II against *Staphylococcus aureus*

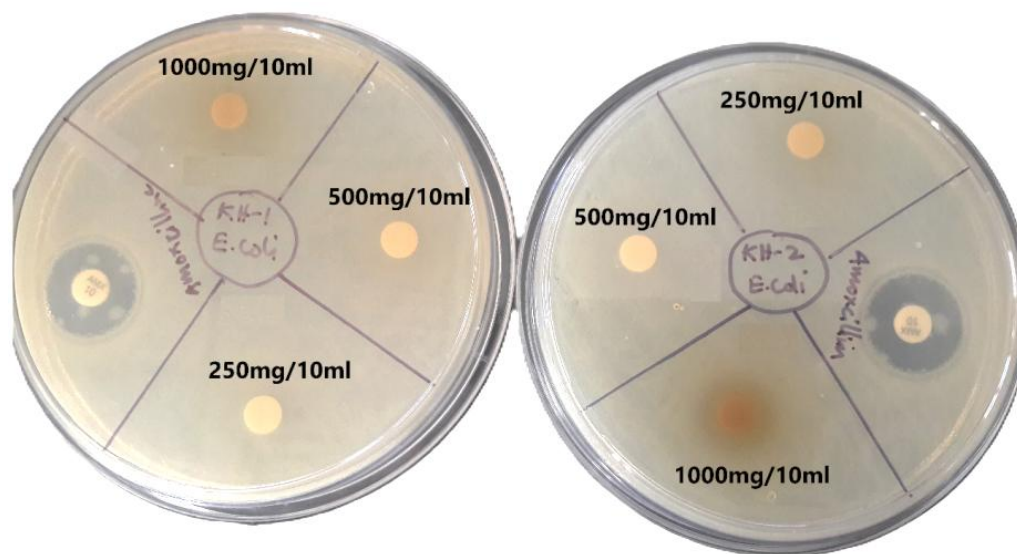


Figure 5. The Appearance of Disc with no Inhibitions

Discussion

Crude extracts of Khyung Inga KH-I and KH-II showed antimicrobial activity using the disc diffusion method, with considerable zones of inhibition against *Staphylococcus aureus*. The KH-II batch produced in 2018 demonstrated slightly stronger effects than the 2016 batch, suggesting that newer batches are more effective and highlighting the expiration dates of traditional medicine.

The study identified A-ru (*Terminalia chebula* Retz.), the key ingredient in KH, as the main driver of the antimicrobial activity in the test medicine. When we tested all five ingredients separately, only *T. chebula* showed real activity against bacteria. This finding is further supported by a review paper in the International Journal of Food Properties, where the authors stress how effective *T. chebula* is at fighting off all sorts of harmful bugs (11).

The findings are further supported by research published in the African Journal of Microbiology Research, which showed that an ethanol extract of *Terminalia chebula* Retzius had strong antibacterial effects against *Staphylococcus aureus*. The minimum inhibitory concentration was 100 mg/mL, and the extract produced a 20 mm zone of inhibition at this concentration, which is comparable to the 15 mm zone produced by the standard amoxicillin 10 µg control.

Additionally, the extract inhibited bacterial growth with zones of 15 mm at 50 mg/mL and 10 mm at 25 mg/mL (12).

The lack of antibacterial activity against Gram-negative bacteria (*E. coli*, *S. typhimurium*) and minimal effect on *P. aeruginosa* aligns with established microbiology principles. Gram-negative bacteria feature an asymmetric outer membrane rich in lipopolysaccharides, which serves as a formidable permeability barrier to hydrophobic phytochemicals, such as those in KH extracts. This contrasts with Gram-positive *S. aureus*, lacking such a membrane and relying on a porous peptidoglycan wall susceptible to disruption. Efflux pumps in Gram-negatives further expel potential antimicrobials, reducing efficacy a pattern consistent with ethanol extracts of *Terminalia chebula* (13).

Terminalia chebula Retzius serves as a primary contributor to the observed antimicrobial activity against *Staphylococcus aureus*, as evidenced by its well-documented antibacterial effects. KH's multicomponent formulation includes four other ingredients, *Aconitum laciniatum*, *Acorus calamus* L., *Saussurea lappa*, and *Delphinium brunonianum*, that may synergistically enhance efficacy. A limitation of this study is the lack of fractionation to isolate individual contributions, precluding definitive attribution of activity; future phytochemical analyses and bioassays on

separated components are recommended to elucidate synergies.

Conclusion

Recent findings suggest that KH demonstrates antimicrobial activity against *Staphylococcus aureus*. This bacterium causes various skin infections, such as abscesses, boils, cellulitis, folliculitis, impetigo, and other rashes. Therefore, KH could be recommended as a complementary treatment for these conditions, potentially reducing the need to import medicines.

However, these results are based on in vitro studies. Further research, particularly in vivo studies, is needed to confirm KH's effectiveness before making clinical recommendations.

Acknowledgment

The authors thank Dr Sonam Wangchuk from RCDC for his valuable administrative support. They also thank Mr. Sangay Dorji, Dorji Tshering, and Ugyen Penjor from the EIDL RCDC for their technical assistance throughout the antimicrobial bioassay. Additionally, the authors are grateful to Mr Samten from MSCPL for his help with sample extractions.

Declaration

Waiver of consent was obtained from REBH via letter No. REBH/PO/2018/00, dated November 17, 2018.

Consent for Publication

Not applicable.

Funding Statement

No funding was received.

Authors' Contribution

Conceptualization, methodology, formal analysis, investigation, resources: JS;

Laboratory investigation and data interpretation: JS, TD;

Writing original draft: JS, ST;

Review, editing of the draft: JS, ST, TD.

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