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EVALUATION OF ANTIMICROBIAL ACTIVITY AND TIME-KILL KINETICS OF CHITHIRAMOOLA KULIGAI AGAINST MICROBIAL PATHOGENS

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ABSTRACT

The vaginal microbiota plays a crucial role in maintaining physiological homeostasis and protecting against pathogenic invasion. Dysbiosis, or imbalance in the vaginal microbiota, is associated with increased susceptibility to sexually transmitted infections like human papillomavirus, which is linked to cervical cancer. Traditional herbal medicine, such as "Chithiramoola Kuligai" (CMK), offers potential as an alternative therapeutic approach, yet its antimicrobial efficacy remains largely unexplored. This study aimed to comprehensively assess the antimicrobial activity of CMK against a range of microbial pathogens, including bacteria and fungi. Time-kill kinetics assays were also conducted to investigate the dynamic interaction between CMK and microbial populations over time. Agar well diffusion assays assessed CMK's susceptibility to diverse microbes. Broth microdilution assays determined Minimum Inhibitory Concentration against individual pathogens. Time-kill kinetics explored CMK's concentration-dependent efficacy and mechanisms. CMK exhibited significant inhibitory activity against both bacterial and fungal strains. The zone of inhibition (ZOI) for CMK ranged from 12mm to 31mm, depending on the strain and concentration. MIC values indicated potent antimicrobial effects, particularly against gram-positive bacteria and *Candida albicans*. For example, the MIC values for *S. aureus* and *E. coli* were 75 mg/ml, while for *L. acidophilus* and *C. albicans*, it was 50 mg/ml. Time-kill kinetics analysis revealed concentration-dependent efficacy, with decreased viable bacterial and fungal counts observed at concentrations equal to or greater than the MIC. CMK exhibits significant antimicrobial activity, supporting its traditional use in infectious disease treatment. Further research is needed to understand its mechanisms and optimize efficacy, indicating CMK's potential as a novel antimicrobial agent.

Keywords: vaginal microbiome, cervical cancer, Siddha, antimicrobial, dose-dependent.

INTRODUCTION

The vaginal microbiota, constituting about 9% of the total human microbiota, maintains a mutualistic relationship with the host, which is crucial for physiological homeostasis and safeguarding against pathogenic invasion.[1,2] The intricate interplay between the host and its resident microbiota, collectively known as the normal vaginal flora, is essential for preserving vaginal health and preventing opportunistic infections. Central to this symbiotic relationship is the predominance of lactobacilli, which contribute to the maintenance of an acidic vaginal pH, the production of antimicrobial compounds, and the competitive exclusion of potential pathogens.[3] The presence of a diverse array of microorganisms within the vaginal milieu serves as a bulwark against colonization by exogenous pathogens, ensuring the preservation of vaginal health. However, disruptions in the delicate equilibrium of the vaginal microbiota are often triggered by both internal and external factors. Hormonal changes, age, sexual practices, immune system, etc., have their ability as internal factors to control the opportunistic pathogens that could invade the human body and cause illness.[4-7] The microbiota balance within the host is influenced by various external factors such as antibiotics, infections, and environmental microbial exposure, potentially increasing the risk of diseases. Disruptions in these internal and/or external factors can lead to an imbalance in the microbial ecosystem, known as dysbiosis.[8] Dysbiosis of the vaginal microbiota, in particular, has been linked to a higher susceptibility to sexually transmitted infections (STIs), including human papillomavirus (HPV) infection.

HPV, a ubiquitous sexually transmitted pathogen, represents a significant public health concern due to its established role in the pathogenesis of cervical cancer.[9-11] Persistent infection with high-risk HPV genotypes, notably HPV types 16 and 18, can lead to the gradual progression of cervical intraepithelial neoplasia (CIN) to invasive cervical carcinoma, underscoring the importance of early detection and prevention strategies.[12] Emerging evidence suggests a complex interplay between HPV infections, alterations in the vaginal microbiota, and the development of cervical cancer. Dysbiotic vaginal microbiota characterized by a decreased abundance of lactobacilli and an overgrowth of pathogenic bacteria has been implicated in promoting HPV persistence, cervical dysplasia, and, ultimately, the progression to cervical cancer.[13]

Moreover, in the global healthcare landscape, the emergence of antimicrobial resistance (AMR) poses a formidable challenge, threatening the efficacy of conventional antibiotics and necessitating the exploration of alternative therapeutic approaches.[14] One such formulation of interest is "Chithiramoola Kuligai" (CMK),[15,16] a herbo-metallic compound traditionally employed in Siddha medicine for managing cervical and reproductive cancers. Beyond its anticancer properties, CMK is also believed to possess antimicrobial activity, although its efficacy and mechanisms of action remain largely unexplored in contemporary scientific research.

This study aims to elucidate the antimicrobial potential of CMK against a diverse array of microbial pathogens. Through a comprehensive series of laboratory experiments and assays, we endeavour to assess the inhibitory and bactericidal/fungicidal effects of CMK on clinically relevant bacterial and fungal strains. The experimental framework encompasses several critical methodologies designed to interrogate different facets of CMK's antimicrobial activity. Initially, agar well diffusion assays are employed to provide a broad assessment of CMK's susceptibility against various microbial strains. Subsequently, broth microdilution assays are utilized to determine the minimum inhibitory concentration (MIC) of CMK, offering insights into its potency against individual pathogens. Furthermore, time-kill kinetics assays are conducted to explore the dynamic interaction between CMK and microbial populations over time, shedding light on its concentration-dependent efficacy and potential mechanisms of action. Through meticulous data analysis and interpretation, we aim to delineate the spectrum of microbial susceptibility exhibited by CMK and its comparative efficacy against standard antimicrobial agents.

By elucidating the antimicrobial properties of CMK through rigorous scientific inquiry, this study seeks to bridge the gap between traditional herbal medicine and modern antimicrobial therapy. By elucidating the antimicrobial efficacy of CMK against a spectrum of microbial pathogens, this study not only contributes to our understanding of traditional herbal medicine but also offers insights into the broader implications of microbiota dysbiosis in the context of HPV-associated cervical carcinogenesis. Through interdisciplinary research efforts, we endeavour to harness the healing potential of ancient remedies to address contemporary healthcare challenges, with a focus on preserving vaginal health and combating cervical cancer.

MATERIALS AND METHODS

Drug details

The experimental medication utilized in this current investigation is known as ″Chithiramoola Kuligai" (CMK), sourced from a reputable pharmaceutical company certified under GMP standards. CMK is a herbal-mineral compound derived from the Siddha system of medicine. It is formulated in pill form, comprising the root bark of *Plumbago zeylanica, Trachyspermum ammi*, purified mercuric chloride, and palm jaggery. The specific batch utilized in the study bears Batch No. S11-135 and was manufactured in September 2022.

Preparation of extract

Initially, a stock solution of CMK was prepared by dissolving a known quantity of CMK powder in a suitable solvent, typically DMSO, to create a concentrated solution. From this stock solution, various concentrations of CMK were generated by diluting the stock solution with distilled water containing 10% DMSO. The specific concentrations of CMK were calculated based on the experimental requirements, and the volumes of the stock solution and diluent were adjusted accordingly to achieve the desired concentrations in the final 1 ml solution.

Culture medium procurement and preparation

Mueller Hinton Agar (MHA) (Sigma-70191) and Sabouraud dextrose agar (SDA) (M1067), both acquired from Sigma-Aldrich, Michigan, USA, were prepared as follows. For MHA, 38 g of the medium was dissolved in 1000 ml of distilled water, followed by boiling until complete dissolution. The solution was then autoclaved at 121°C for 15 minutes for sterilization. For SDA, 65 g of the medium was mixed with 1000 ml of distilled or deionized water and heated to boiling with intermittent shaking for complete dissolution. The solution was autoclaved at 121°C for 15 minutes to ensure sterility.

Collection and preparation of test organisms

To explore microbial characteristics, cultures were sourced from Clinical Laboratories in and around Chennai, encompassing a range of organisms: Three-gram positive bacterial strains, namely, *Staphylococcus aureus* (ATCC-29213), *Lactobacillus acidophilus* (MTCC-10307), *Enterococcus faecalis* (ATCC-29212), and two-gram negative bacterial strains namely *Escherichia coli* (ATCC-25922), *Klebsiella pneumoniae* (ATCC-700603) in addition to a fungal strain, *Candida albicans* (obtained from Clinical Cochin University). Each organism's identity was confirmed via specific biochemical tests delineated in Mackie and McCartney, Practical Medical Microbiology.[17]

A standardized suspension of these organisms was prepared by inoculating a loopful of each culture into 10 ml of nutrient broth, followed by an incubation period at 37ºC for approximately 6 to 8 hours to attain a lag phase consistency. This meticulous process aimed to ensure the reliability and accuracy of subsequent microbial analyses.

Microbial susceptibility assay using agar well diffusion method

In this initial experiment, the antimicrobial screening of CMK was assessed using the Agar well diffusion method. Media preparation involved autoclaving and dispensing into Petri plates. Bacterial and fungal cultures were transferred onto the agar, and wells were created for test substances. CMK extract, diluted to concentrations from 75 to 500 mg/ml, was added to wells, along with standard antimicrobial drugs for comparison. Plates were then incubated at appropriate temperatures (Bacterial cultures at 37 °C and fungal cultures at 30 °C) for 12 to 24 hours. Afterwards, inhibition zones around wells were measured to evaluate microbial growth inhibition by CMK. Each experiment was conducted in duplicate for reliability, following a methodology similar to Balouiri *et al*.[18]

Determination of minimum inhibitory concentration (MIC) using broth microdilution method

The Minimum Inhibitory Concentration (MIC) determination was conducted using the broth microdilution method within 96-well trays, with each experiment replicated three times for reliability. Resazurin, prepared at 0.015%, underwent stringent preparation steps and was stored at 4 °C for stability. Inoculum preparation followed CLSI recommendations, adjusted to a precise concentration of 10^6 CFU ml^{-1} using a calibration curve. Microbial strains were adjusted to 0.5 McFarland standard turbidity and further diluted before addition to wells with diluted samples in Mueller-Hinton broth medium. Concentrations ranging from 100 to 0.32 mg/ml (100, 75, 50, 25, 12.5, 6.25, 3.1, 1.5, 0.75 and 0.32mg/ml) were evaluated across a 96-well tray, allowing comprehensive assessment. Trays were monitored over specified incubation periods, with bacterial strains observed for 12-24 hours at 37 °C and fungus for 48 hours at 30°C. Each well-

received culture media, microbial suspension, and diluted formulation ensures consistency. After incubation, resazurin was added to all wells. Resazurin turning pink indicated bacterial growth, with the MIC defined as the lowest inhibitory concentration preventing this change. The minimum bactericidal/fungicidal concentration (MBC/MFC) was established as the lowest concentration of the formulation, where no bacterial or fungal growth was observed. Experimental procedures were repeated three times for accuracy and validity.[19]

Time-kill kinetics assay

Time-Kill Kinetics analysis of CMK followed the methodology mentioned by Tsuji *et al.* in 2008, with slight modifications.[20] The preparations were set up with concentrations matching the Minimum Inhibitory Concentration (MIC), twice the MIC, and half the MIC of the extracts. A volume of 1.0×10^6 colony-forming units per millilitre (CFU/mL) was introduced and then incubated at 37 °C. At specific time points (0, 2, 6, 12, and 24 hours for bacteria, and 0, 6, 12, 24, and 48 hours for fungi), samples of 1.0 mL were withdrawn from the medium. These samples were carefully transferred into 20 mL of nutrient agar in aseptic conditions, followed by incubation at 37 °C for 24 hours. Concurrently, control tests were conducted on the organisms without the CMK sample.

After incubation, the organisms' colony-forming units (CFU) were quantified. This entire procedure was independently repeated three times. Subsequently, the data obtained from the experiments were used to plot a graph illustrating the logarithm of colony-forming units per millilitre (CFU/mL) against time.[21]

Statistical analysis

Experiments were repeatedly performed, and the results were plotted as mean ± SD using MS Excel, and a graph was created using Origin Pro3.

Table 1: Zones of Growth Inhibition of CMK and standard drugs against test organisms

Samples	Concentrations	Zone of inhibition in mm Test Organisms					
		SA	LA	EF	KP	EC	CA
***Chithiramoola Kuligai* (CMK)**	75	12	14	14	-	14	18
	150	15	16	16	-	16	22
	250	17	21	16	-	17	25
	300	18	22	18	-	17	28
	500	20	23	20	-	19	31
Ampicillin (Amp)	10 mcg/ml	12	24	10	ND	ND	ND
Norfloxacin (Nx)	10 mcg/ml	ND	ND	ND	24	21	ND
Amphotericin (Ap)	20 mcg/ml	ND	ND	ND	ND	ND	11

Zones of growth inhibition = diameter of well + zone of growth inhibition; diameter of well = 8mm. SA = *Staphylococcus aureus*; LA = *Lactobacillus acidophilus;* EF = *Enterococcus faecalis;* KP = *Klebsiella pneumonia;* EC = *Escherichia coli;* CA = *Candida albicans.* ND= *Not Determined.*

Table 2: MIC, MBC and MFC of CMK against susceptible organisms

Test sample	Activities	*S. aureus* (mg/ml)	*L. acidophilus* (mg/ml)	*E. faecalis* (mg/ml)	*E. coli* (mg/ml)	*C. albicans* (mg/ml)
CMK	MIC	75.0 ± 0.25	50.0 ± 0.54	50 ± 0.1	75 ± 0.1	50 ± 0.0
	MBC/MFC	100± 0.01	100 ± 0.03	100± 0.00	100± 0.15	50± 0.0

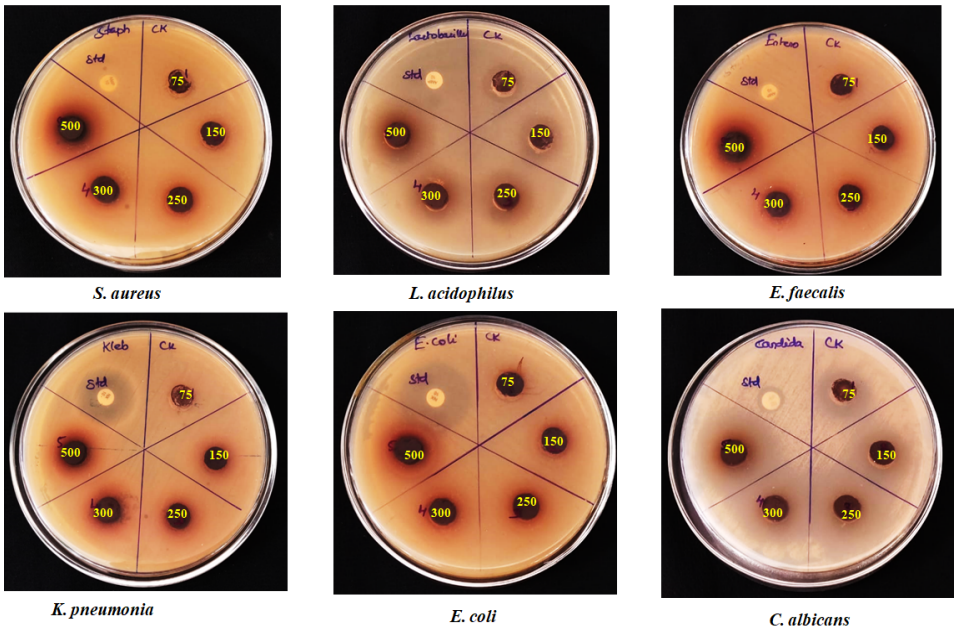

Figure 1: Petri-Dishes showing Zones of Growth Inhibition of CMK and Standard Drugs against test organisms

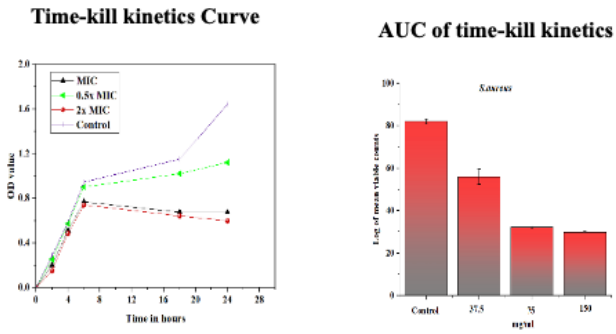

Figure 2A: *Staphylococcus aureus*

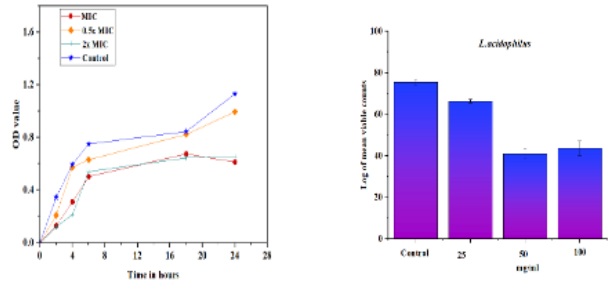

Figure 2B: *Lactobacillus acidophilus*

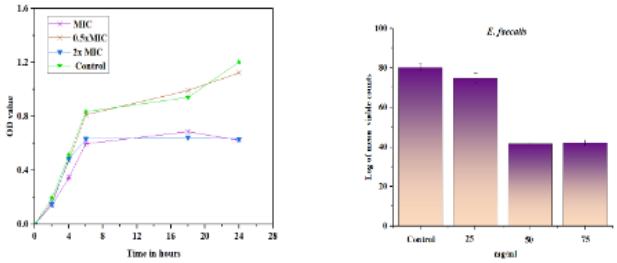

Figure 2C: Enterococcus faecalis

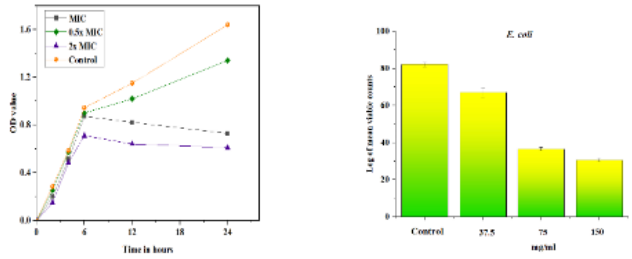

Figure 2D: *Escherichia coli*

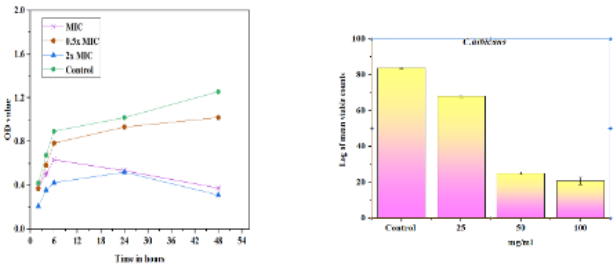

Figure 2E: *Candida albicans*

Figure 2A-2E: Time-kill kinetic curve and Area under Curve (AUC) of time-kill kinetics of test organism against CMK

RESULTS

Antimicrobial susceptibility of CMK

The CMK extract demonstrated varying degrees of zone of inhibition, ranging from 12mm to 23mm, against different gram-positive bacterial strains. Even at its most diluted concentration, the experimental drug showed superior effectiveness compared to the standard drug (Amp.), with respective zone of inhibition measurements of 12 mm and 10mm against *S. aureus* and *E. faecalis*. Against *E. coli*, a gram-negative strain, the zone of inhibition ranged from 14 mm to 19 mm, although it showed comparable results to the standard drug (Nx), which had a 21mm zone of inhibition. However, it's important to highlight that *Klebsiella spp*. exhibited resistance to CMK, suggesting its limited effectiveness against this specific pathogen.

Conversely, in the case of the fungal strain *C. albicans,* the CMK extract displayed the highest range of zone of inhibition, spanning from 18 mm to 31 mm. Even at its lowest concentration, CMK demonstrated significant growth inhibition compared to the standard drug (Ap), which had an 11mm zone of inhibition. Overall, CMK exhibited notable inhibitory activity against both bacterial and fungal strains, as outlined in Table 1 and depicted in Figure 1. This underscores the potent antimicrobial efficacy of the Siddha formulation, which is particularly evident in specific strains where significant growth inhibition was observed.

Determination of MIC, MBC and MFC of CMK

The research revealed exciting insights into the inhibitory effects of the tested formulation, CMK, on various microorganisms. The minimum inhibitory concentrations (MIC) observed on *L. acidophilus, E. faecalis, and C. albicans* were found to be 50 mg/ml. However, for *S. aureus and E. coli*, the MIC was determined to be 75 mg/ml, indicating that the formulation exhibits more significant activity against gram-positive organisms than gram-negative ones. Moreover, it demonstrated significant inhibitory effects on *C. albicans*. These findings are summarized in Table 2.

Time-kill kinetics assay

The time-kill kinetics analysis of CMK against *S. aureus* demonstrated an initial rise in colony count up to 6 hours, followed by a gradual decrease or stabilization until 24 hours compared to the untreated control (Figure 2A). Similarly, for *L. acidophilus*, there was a gradual increase in colony count until 6 hours, followed by a plateau until 18 hours. Beyond this point, the 50 mg/ml (MIC) and 100mg/ml (2MIC) concentrations maintained a stable or decreasing trend until 24 hours, while lower concentrations and the control showed an increase in colonies (Figure 2B). *E. faecalis* showed a significant increase in colony count up to 6 hours, remaining constant at the highest concentration until 24 hours. There was a slow increase until 18 hours at the MIC concentration, followed by a decrease, whereas the control and lower concentrations exhibited continuous growth (Figure 2C). Against *E. coli,* there was an increase in colony count until 6 hours, with subsequent gradual decreases observed at higher concentrations (Figure 2D). For *C. albicans*, the control and lowest concentration showed a gradual rise until 24 hours, while higher concentrations exhibited a noticeable decline in growth (Figure 2E). Overall, viable bacteria significantly decreased at twice the MIC concentration after 24 hours, and *C. albicans* at 50 mg/ml showed no viable colonies after 24-48 hours, indicating CMK's efficacy against fungal strains. Minimal colony counts were observed for other bacterial strains after 24 hours.

DISCUSSION

The health of the vaginal ecosystem is intricately linked to overall human health and reproductive outcomes.[22] Maintaining vaginal health relies on the delicate balance of indigenous microbiota, which is crucial for fending off opportunistic infections. Dysbiosis, or imbalance, in this microbiota, raises concerns, especially regarding susceptibility to sexually transmitted infections (STIs) like human papillomavirus (HPV), notably types 16 and 18, which can progress reference to cervical cancer. Changes in the cervicovaginal microbiome and processes, including bacterial vaginosis, cervical inflammation, and increased vaginal pH, all affect susceptibility to cervical HPV.[23] HPV significantly contributes to cervical cancer and precancerous lesions, but it's not the sole cause.[12] Its persistence is influenced by factors like imbalanced cervicovaginal microbiota and inflammation. The damaged epithelial barrier facilitates HPV infection by creating optimal conditions.[24] Persistent HPV infection impacts cervical and vaginal microbiology, compromising host immune defences.[25] HPV can also alter mucosal metabolism, affecting the cervix-vaginal microenvironment. It triggers inflammation-related mechanisms, activating macrophages, NK cells, and local mucosal immunity. HPV, bacterial vaginitis, and microbiota dysregulation mutually influence each other, complicating the scenario of cervical cancer development.[26] Dysbiosis can induce several characteristics of cancer, such as barrier disruption, abnormal cellular proliferation, genomic instability, angiogenesis, chronic inflammation, and dysregulation of metabolism.[27] The interaction of HPV, cervicovaginal microbiota, and inflammation emphasizes the need to maintain a balanced vaginal ecosystem for effective cervical cancer prevention and treatment. Prioritizing interventions to restore microbiome balance and bolster immune responses is crucial. Additionally, the limitations of conventional antibiotics highlight the urgency of exploring alternative therapies for early detection and prevention.

In response to the growing need for alternative therapies, CMK, a traditional Siddha medicine, has garnered attention for its potential in managing cervical and reproductive cancers. Despite containing ingredients with inherent antimicrobial properties,[28,29] CMK's efficacy and mechanisms against microbes remain primarily unexplored in scientific research. Therefore, this study seeks to comprehensively assess CMK's antimicrobial activity by investigating its susceptibility, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum fungicidal concentration (MFC), and conducting time-kill kinetics assays. These experiments provide crucial insights into CMK's potential as an antimicrobial agent against various pathogens, contributing to our understanding of its therapeutic properties.

The observed zone of inhibition (ZOI) against different bacterial and fungal strains highlights the broad-spectrum antimicrobial activity of CMK. The difference in the diameter zone of inhibition for CMK at the lowest and highest concentration (Table 1) may result from the effect of diffusion of the bioactive agents within the medium.[30] Among the gram-positive bacterial strains, CMK inhibited the growth of all the selected strains very well. CMK exhibited superior potential against *S. aureus*, *E. faecalis and E. coli* bacterial strains compared to standard drugs, consistent with previous studies on *Plumbago zeylanica.*[31-33] Kaur *et al*. demonstrated that *Trachyspermum ammi* exhibits antibacterial activity against *S. aureus and E. faecalis*, albeit with a lesser effect against *K. pneumonia.*[34]

CMK's resistance against *K. pneumoniae* aligns with the findings of Jeyachandran *et al*. and Kaur *et al*.[34,35] Mercurial compounds, as found in CMK, have been shown to exhibit antibacterial activity.[36] Palm jaggery, a significant component of CMK, is rich in nutrients like potassium, ascorbic acid, and Vitamin B12, which may contribute to its antimicrobial properties. Research on ascorbic acid has shown its effectiveness in inhibiting the growth of both gram-positive and gram-negative bacterial strains, including *S. aureus, E. coli,* and *K. pneumonia.*[37]

Compared to bacterial strains, CMK effectively inhibits the growth of C. albicans, likely due to the antifungal properties of the raw ingredients in the formulation.[38,39] MIC results indicate that CMK is particularly potent against *L. acidophilus, E. coli,* and *C. albicans,* consistent with previous studies. While it is commonly believed that species of *Lactobacillus sp.* dominate healthy vaginal flora, recent research suggests that several anaerobic bacteria may be predominant, challenging this notion. Further exploration of other *Lactobacillus* variants holds promise for captivating research.[40,41]

The time-kill kinetics assay provides dynamic information on the antimicrobial activity of CMK over time. The observed decrease in viable bacterial and fungal counts at concentrations equal to or greater than the MIC indicates fungicidal activity against *C. albicans* and bacteriostatic activity against bacterial strains. This is consistent with the concept of concentration-dependent killing, where higher concentrations of antimicrobial agents lead to more rapid and extensive microbial eradication. These findings contradict those of Emmanuel *et al.,* where plumbagin demonstrated fungistatic activity against *C. albicans*. The use of crude root in CMK preparation may contribute to its fungicidal activity.[42]

CONCLUSION

Overall, the results suggest CMK possesses significant antimicrobial activity against various pathogens, particularly gram-positive bacteria and *C. albicans*. These findings support the traditional use of CMK in treating various infectious diseases and highlight its potential as a novel antimicrobial agent. Further studies are warranted to elucidate the underlying mechanisms of action and optimize its therapeutic efficacy.

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