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# Antibacterial Effects of *Calendula officinalis* Preparations on South African ESKAPE Pathogens

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ABSTRACT

**Background:** Medicinal plants possess considerable potential for discovering new phytochemicals that could be considered as a solution to fight against multidrug-resistant pathogens. *Calendula officinalis* (C. officinalis) is used worldwide due to its antimicrobial properties. This pilot study assessed the antibacterial activity of herbal extract and homeopathic preparation of *C. officinalis* flowers against South African ESKAPE pathogens. .

**Materials & Methods:** Kirby-Bauer disc diffusion method (with a 6.0 mm disk diameter) was employed to evaluate the antibacterial activity of herbal extract and homeopathic preparation against South African ESKAPE pathogens. Various ethanol concentrations of herbal extract (50, 60, and 90%) and 62% ethanol concentration of homeopathic preparation were tested.

**Findings:** The inhibitory effect of *C. officinalis* did not surpass that of antibiotics. However, the ethanol herbal extract of *C. officinalis* showed some antibacterial activity against ESKAPE pathogens compared to its homeopathic preparation. Moreover, 50% ethanol extract of *C. officinalis* (20 µL) showed significant antibacterial activity against *Staphylococcus* species compared to its homeopathic preparation.

**Conclusion:** The rapid spread of antibiotic resistance necessitates the search for plant-based antibacterials. Due to their wealth in phytochemicals, medicinal plants provide a rich resource for producing novel antibacterial drugs. The current study attempted to demonstrate the inhibitory activities of ethanol herbal extract (HEs) and homeopathic mother tincture (MT) of *C. officinalis* flowers against ESKAPE pathogens and *Escherichia coli* species.

**Keywords:** *Calendula officinalis*, Multidrug resistance, Kirby-Bauer disk-diffusion method, Herbal extract, Homeopathy

CITATION LINKS

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## Introduction

Medicinal plants are widely used as remedies to cure, prevent, and manage various ailments worldwide. This is due to their easy accessibility, affordability, and less side effects, which are perceived by communities [1]. Multidrug resistance remains a challenging health care problem in developed and developing countries. This phenomenon has prompted a shift towards finding medicinal plants as alternative antibiotics. Pot marigold (*C. officinalis*) is a plant historically used in traditional medicine (TM). The World Health Organisation (WHO) [2] explains TM as "the sum of knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as prevention, diagnosis, and improvement/ treatment of physical and mental illness". *C. officinalis* is a flowering plant classified under the daisy family *Asteraceae*. It possesses various chemical constituents that contribute to its medicinal use [3]. *C. officinalis* is commonly used in homeopathy and traditional medicine as an antiseptic remedy in the form of extract, tincture, or balm to heal skin wounds and treat skin conditions [4]. This plant has also been found to possess antibacterial, anti-tumour, anti-fungal, antiviral, anti-inflammatory, and antioxidant activities due to the presence of numerous secondary metabolites [5]. Research has shown that *C. officinalis* contains secondary metabolites and phytochemicals in multiple parts of the plant, including triterpenes, saponins, triterpendiol esters, polysaccharides, steroids, tannin, quinines, coumarins, flavonoids, carotenoids, amino acids, and essential and volatile oils [1, 4, 5]. These secondary metabolites are accepted and confirmed to be responsible for the antimicrobial activities of this plant [6]. Additionally, the literature has proved that *C. officinalis* has antimicrobial potential against Gram-negative and Gram-positive pathogens [7, 8]. Using different extraction methods, *C. officinalis* metabolites have been added to different herbal formularies to treat many conditions. Studies have shown that the effectiveness of herbal extracts (HEs) depends on their ability to deliver therapeutic active compounds [9]. HEs are sources of biologically active constituents with therapeutic effects. In order for HEs to effectively exert their therapeutic and medicinal effects, their preparation method must comply with manufacturing standards [10]. A homeopathic preparation, mother tincture (MT), is a crude botanical extract of fresh or dried plants, prepared according to the guidelines in the Homeopathic Pharmacopoeia. Mother tinctures are used as the starting materials for homeopathic dilutions [11, 12].

In the past decade, the medical world has witnessed the increasing prevalence of multidrug-resistant (MDR) ESKAPE pathogens (*Enterococcusfaecium, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Acinectobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species), which has increased the burden of disease and reduced treatment options for infections [13]. Additionally, a review study by Founou and colleagues (2017) [14] reported a significant association between ESKAPE pathogens and the highest risk of mortality and health care costs. According to the WHO [15], it is expected that in 2050, 10 million people will die due to antimicrobial resistance (AMR), and 100 trillion USD of the world's economic output will be lost because of this health threat. ESKAPE pathogens cause several diseases and could escape the biocidal effects of antimicrobial agents [16]. In particular, Gram-positive bacteria such as *E. faecium* and *S. aureus* cause endocarditis, bacteremia, sepsis, and pneumonia, and

Gram-negative bacteria such as *A. baumannii, K. pneumoniae, P. aeruginosa, Enterobacter* spp., and *E. coli* commonly cause urinary tract and respiratory tract infections [17, 18]. These pathogens are associated with hospital-acquired infections worldwide [19, 20]. In a country like South Africa, where there are challenges in the healthcare system, such as lack of health resources, the emergence of infectious diseases continues to be a problem in hospitals and the community [14, 21]. Although substantial research has been conducted on *C. officinalis* (HE), little research is available on the antimicrobial potency of *C. officinalis* against resistant South African Gram-positive and Gram-negative strains. This pilot study aimed to identify new and alternative antibacterial treatments for South African ESKAPE pathogens.

**Objectives:** While several studies have reported the potential of herbal medicines as alternative antibiotics, to the best of the researcher's knowledge, no research has been conducted to investigate their effects on South African ESKAPE pathogens. Therefore, it is important to identify and test indigenous medicinal plants in South Africa, which are effective in eradicating resistant pathogens. This study serves as a baseline for future studies and interventions designed to eradicate ESKAPE pathogens.

## Materials and Methods

**Study design:** This *in-vit*ro quantitative control study was conducted at the Complementary Research Facility (CMRF) of the University of Johannesburg (UJ) in January 2022.

**Study population Antimicrobial agents**: The herbal extract (HEs) and homeopathic mother tincture (MT) of *C. officinalis* flowers were obtained from different reputable companies based in South Africa. These companies follow good manufacturing practices and issue certificates of analysis (CoA). *C. officinalis* HE was prepared in different ethanol concentrations (50, 60, and 90%) following the relevant monographs available in the British Pharmacopoeia (BP) [22]: 50, 60, and 90% herbal extracts (HEs) were prepared in dilutions of 1.10 (batch no. 6412), 1:5 (batch no. 6412), and 1:2 (batch no.171221), respectively. Also, homeopathic MT was manufactured in 62% ethanol according to the German Homeopathic Pharmacopoeia (GMP) [23] in a dilution of 1:10 (batch no. C23019).

**Antibacterial strains:** South African ESKAPE strains and *E. coli* clinical isolates were obtained from the University of Johannesburg (UJ) Water and Health Research Centre (WHRC) culture bank. Strain verification and drug resistance screening were performed on these clinical isolates using the VITEK®2 Compact System (BioMérieux, USA) according to Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines [24]. To ensure the validity of the results obtained, ATTC-authenticated reference ESKAPE and *E. coli* strains were included in all experiments, including *E. faecium* ATTC2720, *S. aureus* ATCCBAA-1026, *K. pneumoniae* ATCC13883, *A. baumannii* ATCC19606, *P. aeruginosa* ATCC10145, *E. coli* ATCC35218, and *E. coli* ATCC25922. All reference strains and clinical isolates were stored at -80 °C as glycerol stocks and grown on Mueller-Hinton agar plates overnight (18-16 hours) at 35 °C.

**Antimicrobial control agents:** Conventional antibiotics and ethanol in different concentrations used as diluents of *C. officinalis* were used as controls. Antibiotic discs containing piperacillin/tazobactam (TZP: 110 mg, CT0725B OXOID), ciprofloxacin (CIP: 5 µg, CT0425B OXOID), and vancomycin and imipenem (VAN/IMP: 30 µg, CT0466B OXOID) were purchased from Sigma Aldrich (Supelco PHR1167).

**Disc diffusion method:** Fresh bacterial cultures for each experiment were prepared by streaking bacteria on Mueller-Hinton (MH) agar culture plates and incubated at 35 °C for 24 hours. First, bacterial suspensions were prepared with MH broth media and bacteria. The concentration of bacterial suspensions was adjusted to 0.5 McFarland standard using bioMérieux densiCHEK plus to ensure consistent results. Then the adjusted bacterial suspensions were spread on MH agar plates and incubated at 35 °C for 24 hours to create bacterial lawns. The plates were then allowed to dry for three to five min before placing the sterile antimicrobial discs on them. The experiment was conducted over three days to ensure the reliability, reproducibility, validity, and accuracy of the results. Sterile discs were impregnated with 20 µL of *C. officinalis* HE (50, 60, and 90%) and homeopathic MT (62%) and placed on agar plates. Appropriate controls (5 µL of antibiotics and 20 µL of ethanol in the mentioned concentrations) were also used and measured. Each MH agar plate was numbered before incubation. The plates were inverted and incubated at 35 °C for 16 to 18 hours. Thereafter, the plates were studied, and the growth inhibition zone diameter (in mm) was measured using an Interscience: Scan® 1200 colony counter from a programmed minimum sub-zero baseline point to a 6-mm (inhibition zone) distance from the disk.

## Findings

In this study, 20 µL of HEs (in 50, 60, and 90% ethanol) and 20 µL of homeopathic MT (in 62% ethanol) of *C. officinalis* flowers were tested individually against ESKAPE pathogens, including *E. faecium* ATTC2720, *S. aureus* ATCCBAA-1026, *K. pneumoniae* ATCC13883, *A. baumannii* ATCC19606, *P. aeruginosa* ATCC10145, *E. coli* ATCC35218, and *E. coli* ATCC25922, respectively. The HEs and homeopathic MT showed varying levels of efficiency against Gram-positive and Gram-negative pathogens (Tables 1 and 2).

**Table 1)** Antibacterial activity of different concentrations of *C. officinalis* HE against clinical bacterial pathogens

| Bacteria | Antibacterial activity of 20 µL *of Calendula officinalis* herbal extract in terms of inhibition zone (IZ) diameter in mm | | |
|---|---|---|---|
| | 20 µL of HE/5 µL of antibiotic (IMP, CIP, TZP)/20 µL of ethanol control | | |
| | 50% Herbal Extract | 60% Herbal Extract | 90% Herbal Extract |
| *A. baumannii* | 8.2mm/30.2mm (IM)/ 6.0mm | 10.0mm/32.5mm (IMP)/11.2mm | 14.1mm/30.6mm (IMP)/16.4mm |
| *Enterobacter spp* | 6.8mm/31.1mm (IMP)/ 6.0mm | 9.5mm/32.3mm (IMP)/6.0mm | 16.2mm/30.2mm (IMP)/14.5mm |
| *E. faecium* | 9.1mm/30.5mm (IMP/ 6.0mm | 9.5mm/28.5mm (IMP)/6.0mm | 15.0mm/27.4mm (IMP)/14.8mm |
| *K. pneumoniae* | 6.0mm/35.6mm (CIP)/ 6.0mm | 7.2mm/37.9mm (CIP)/12.6mm | 15.4mm/34.8mm (CIP)/10.8mm |
| *P. aeruginosa* | 8.4mm/29.2mm (TZP)/6.0mm | 24.4mm/32.6mm (TZP)/10.3mm | 16.4mm/28.5mm (TZP)/12.0mm |
| *S. aureus* | 6.0mm/34.0mm (CIP)/ 6.0mm | 9.1mm/34.2mm (IMP)/9.1mm | 16.2mm/33.9mm (CIP) 16.2mm |
| *E. coli 1* | 6.0mm/29.4mm (IMP)/ 6.0mm | 6.0mm/29.7mm (IMP)/11.0mm | 6.0mm/22.3mm (IMP)/18.8mm |
| *E. coli 2* | 6.0mm/23.3mm (IMP)/ 6.0mm | 6.0mm/34.8mm (IMP)/10.1mm | 6.0mm/24.9mm (IMP)/19.0mm |

**Table 2)** Antibacterial activity of *C. officinalis* homeopathic MT against clinical bacterial pathogens

| Bacteria | Antibacterial activity of 20 µL of *Calendula officinalis* mother tincture in terms of inhibition zone (IZ) diameter in mm | |
|---|---|---|
| | 20 µL of MT/5 µL of antibiotic (IMP, CIP, TZP)/ 20 µL of ethanol | |
| | 62% MT / antibiotic | 62% Ethanol |
| *A. baumannii* | 10.8mm/30.6mm (IMP) | 11.2mm |
| *Enterobacter spp* | 12.0mm/30.2mm (IMP) | 6.0mm |
| *E. faecium* | 19.6mm/27.4mm (IMP) | 6.0mm |
| *K. pneumoniae* | 13.6mm/34.8mm (CIP) | 12.6mm |
| *P. aeruginosa* | 12.7mm/28.5mm (TZP) | 10.3mm |
| *S. aureus* | 10.7mm/33.9mm (CIP) | 9.1mm |
| *E. coli 1* | 10.8mm/33.9mm (IMP, CIP, TZP) | 11.0mm |
| *E. coli 2* | 12.2mm/35.0mm (IMP, CIP, TZP) | 10.1mm |

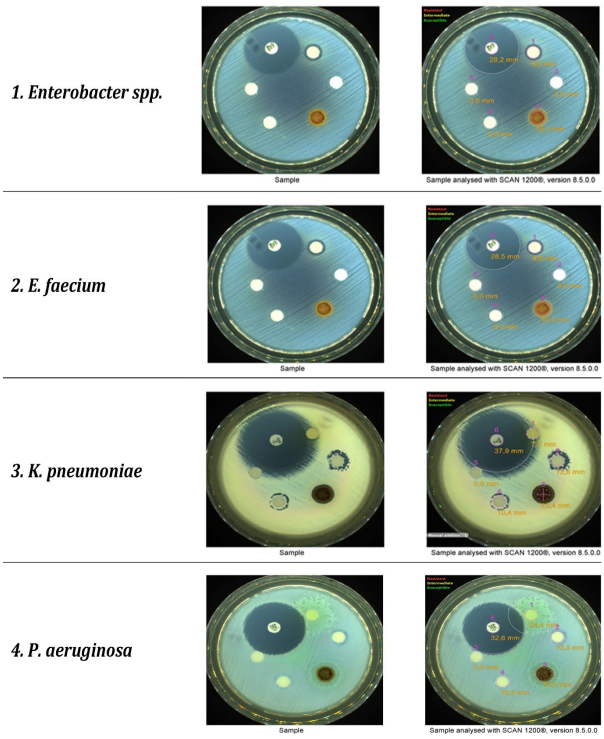


**Figure 1)** Agar plates exhibiting the growth inhibition zones by 20 µL of *C. officinalis flower* 90% HE against ESKAPE pathogens. The central spot represents the positive control (5 µL of antibiotic: IMP, CIP, and TZP), the dark spot stands for *C. officinalis* extract, and the white spot stands for the negative control. 1) *Enterobacter* spp., 2) *E. faecium,* 3) *K. pneumoniae*, and 4) *P. aeruginosa*

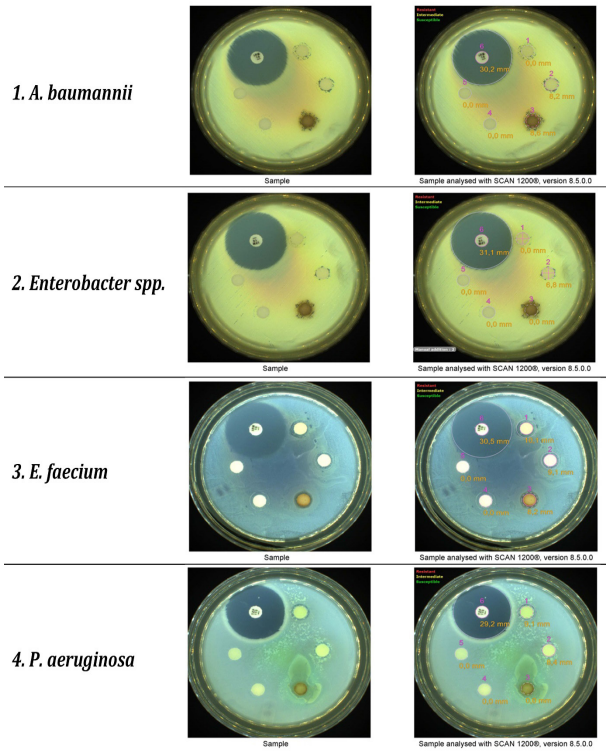


**Figure 2)** Agar plates exhibiting the growth inhibition zones by 20 µL of *C. officinalis flower* 50% HE against ESKAPE pathogens. The central spot represents the positive control (5 µL of antibiotic: IMP, CIP, and TZP), the dark spot stands for *C. officinalis* extract, and the white spot stands for the negative control. 1) *A. baumannii,* 2) *Enterobacter* spp., 3) *E. faecium,* and 4) *P. aeruginosa*.

According to the results, *C. officinalis* HE (20 µL) in 90% ethanol showed greater inhibitory activity than in 50 or 60% ethanol (Figure 1). The 50% HE of *C. officinalis* as a South African-made HE was further tested against 12 *Staphylococcus* species. It showed inhibitory activity against ESKAPE pathogens (Figure 2) and some inhibitory activity against certain *Staphylococcus* spp. (Table 3 and Figure 3).

**Table 3)** Antibacterial activity of 20 µL of *C. officinalis* extract (50%) against different *Staphylococcus* spp.

| *Staphylococcus* Species | *Calendula officinalis* 50% HE | Ethanol 50% | Blank control | Antibiotics (IMP, CIP, and TZP) |
|---|---|---|---|---|
| *S. epidemidis* | 6.0mm | 6.0mm | 6.0mm | 34.0mm |
| *S. capitis* | 7.2mm | 6.7mm | 6.0mm | 31.1mm |
| *S. hominis* | 6.0mm | 0.0mm | 6.0mm | 35.2mm |
| *S. xylosus* | 8.9mm | 6.0mm | 6.0mm | 35.4mm |
| *S. sciuri* | 6.0mm | 6.0mm | 6.0mm | 24.7mm |
| *S. auriculari* | 8.2mm | 6.0mm | 6.0mm | 20.4mm |
| *S. aureus* | 6.0mm | 6.0mm | 6.0mm | 33.5mm |
| *S. caprae* | 6.0mm | 6.0mm | 6.0mm | 33.4mm |
| *S. cohnii* | 6.0mm | 6.0mm | 6.0mm | 31.3mm |
| *S. warneri* | 6.0mm | 6.0mm | 6.0mm | 30.7mm |
| *S. saprophyticus* | 8.2mm | 7.8 mm | 7.2mm | 27.0mm |
| *S. haemolyticus* | 8.4mm | 8.0mm | 7.4mm | 27.1mm |

*S. epidermidis* (ATCCP19), *S. capitis* (ATTCPET20), *S. hominis* (ATCCP13), *S. xylosus* (ATCCBB33592), *S. caprae* (ATCCNP7B7), *S. cohnii* (ATCCJR3X), *S. warneri* (ATCCKS18A), *S. saprophyticus* (ATCCKS18A), and *S. haemolyticus* (ATCCKS16).

## Discussion

This study aimed to evaluate the antibacterial effects of herbal extract (HE) and homeopathic mother tincture (MT) of *C. officinalis* flowers in various ethanol concentrations. The Kirby-Bauer disc diffusion method results showed that the tested *C. officinalis* flower extracts had antibacterial activity against Gram-positive and Gram-negative bacteria. Although the current study used only the disc diffusion method, these results could be a baseline for distinguishing medicinal plants with potential antibacterial activities. The HEs and homeopathic MT of *C. officinalis* flowers showed some antimicrobial potential against Gram-positive and Gram-negative pathogens (Tables 1 and 2). *C. officinalis* (20 µL) HE in 90% ethanol displayed greater antimicrobial activity than in 50 or 60% ethanol. The growth inhibition zone diameter of bacterial isolates was as follows: *E. faecium*: 15.0 mm, *S. aureus*: 16.2 mm, *K. pneumoniae*: 15.2 mm, *A. baumannii*: 14.1 mm, *P. aeruginosa*: 16.4 mm, and *Enterobacter* spp.: 16.2 mm. No inhibitory activity was observed against *E. coli* (6.00 mm) species (Figure 1). According to the WHO [2], *A. baumannii* and *P. aeruginosa* are classified in the critical priority group, and *E. faecium* and *S. aureus* are classified in the high priority group, highlighting the need to develop unexplored medicinal plants [13]. These findings are supported by similar studies documenting the significant inhibitory activities of ethanol extracts of *C. officinalis* against *E. coli, P. aeruginosa*, *Enterococcus* spp., and *Staphylococcus* spp. [4, 25, 26]. The results indicate that 90% ethanol as an effective extraction solvent could significantly influence the efficiency of phytochemicals and secondary metabolites in HE [27]. Moreover, these findings could be attributed to the fact that ethanol extracts of flowers are rich in alkaloids, saponins, and tannins with potent antimicrobial properties [28]. A study by Nouri and colleagues (2021) [29] demonstrated that 90% ethanol extracts of flowers showed significant antibacterial activities against Gram-positive and Gram-negative pathogens. A review study [19] confirmed that ethanolic extracts had the highest antibacterial activities compared to other organic solvents. The homeopathic MT (20 µL) in 62% ethanol showed some

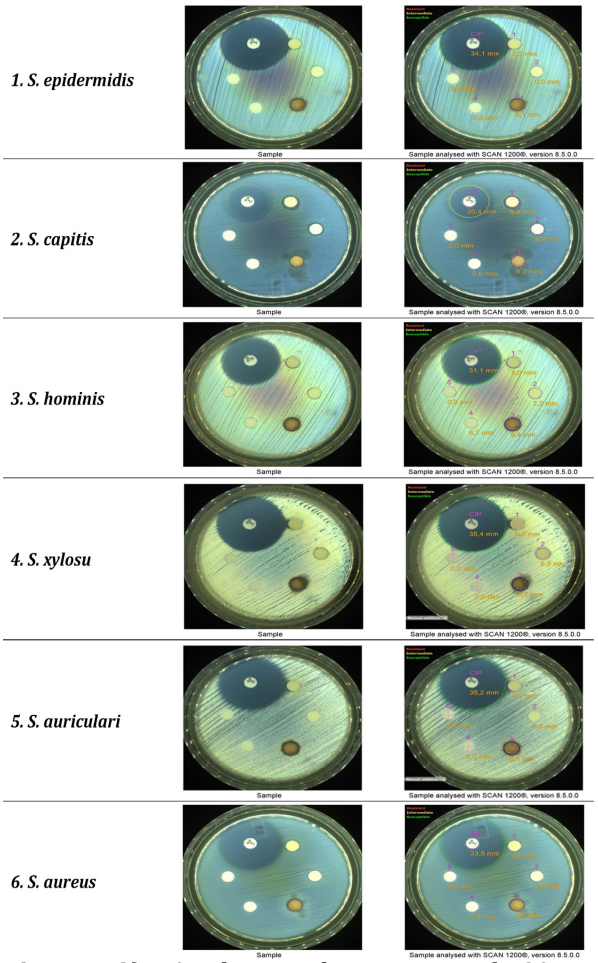


**Figure 3)** Antibacterial activity of 20 µL of *C. officinalis* 50% HE against different *Staphylococcus* species. The central spot represents the positive control (5 µL of antibiotic: IMP, CIP &TZP), the dark spot stands for *C. officinalis* extract, and the white one represents the negative control. 1) *S. epidermidis,* 2) *S. capitis*, 3) *S. hominis,* 4) *S. xylosu*, 5) *S. auriculari*, 6) *S. aureus*

potential to inhibit ESKAPE pathogens, including *E. faecium* (19.6 mm) and *S. aureus* (10.7 mm) (Table 2). This finding suggests that despite the controversy around homeopathic remedies, it is essential to investigate the role of homeopathic MTs as an alternative treatment for ESKAPE pathogens. A study by Jyotisree et al. (2020) [30] suggested *C. officinalis* MT as a promising antibacterial agent. These findings are supported by another study in 2019 [31], where *C. officinalis* MT in 70% ethanol was shown to possess antibacterial properties against *E. coli, S. aureus,* and *P. aeruginosa in vitro*. Although HE and MT did not surpass the antibiotics, they showed some zones of inhibition. This study tested only 20 µL of HE and MT. If different volumes were tested, the results might have shown larger inhibition zones. A study by Shaffique et al. (2020) [32] showed that increasing the volumes of HE and MT affected concentration-dependent inhibition zone. Ethanol control concentrations were also tested, and 50% ethanol showed no zone of inhibition (6.0 mm), whereas 60, 62, and 90% ethanol showed a zone of inhibition more than 6 mm (Tables 1 and 2). This is not surprising since ethanol is known for its ability to kill a broad spectrum of microorganisms, denature proteins, and eradicate bacterial biofilms [33]. This is evident in its use in ethanol lock therapy (ELT) to eradicate and kill central line–associated bloodstream infections (CLABSIs), including *S. aureus, S. epidermis, P, aeruginosa, E. coli,* and *K. pneumonia* [34, 35]. The HE and homeopathic MT were extracted with ethanol according to the German Homeopathic Pharmacopoeia and British Pharmacopoeia. Manufacturers of medicinal plants usually use ethanol as a solvent because it makes the end products safe for consumers.

The results indicate that 50% HE of *C. officinalis* has potential inhibitory activity and is not an organic solvent (Figure 2). The susceptibility variation observed in the tested pathogens could be due to the difference in the permeability of their cell membranes to HEs and MTs. Commercially available antibiotics tested as positive controls showed high inhibition zones against all tested bacterial species (Tables 1 and 2). These results were expected because antibiotics have a better ability to penetrate the biofilm cells of pathogens [36].

In this study, the inhibitory effect of 50% HE of *C. officinalis* was tested on 12 *Staphylococcus* spp., although no inhibitory

activity was observed against *S. aureus*. The reason behind testing only the 50% HE was to evaluate the inhibitory effect of South African *C. officinalis* against South African species.

The findings showed that *C. officinalis* exhibited the highest inhibitory activity against *S. xylosus* (8.9 mm), *S. haemolyticus* (8.4 mm), S. *saprophyticus* (8.2 mm), and *S. auricular* (8.2 mm), but no inhibitory activity was shown against *S. epidermidis*, *S. hominis, S. sciuri, S. aureus, S. caprae, S. cohnii,* and *S. warneri* (6 mm).

The 50% ethanol as a control showed no inhibitory effect, except for *S. saprophyticus* (7.8 mm) and *S. haemolyticus* (8.0 mm) (Table 3 and Figure 3).

*S. aureus* is one of the pathogens causing various infections in healthcare facilities, such as life-threatening endocarditis [37]. Although *Staphylococcus* spp. are harmless inhabitants of the microbiota, each of them could be a potential threat and transfer antibiotic resistance genes to more pathogenic species and intensify their ability to resist drugs [38, 39]. A similar study by Sahingil (2019) [40] found that *C. officinalis* had some inhibitory activities against *S. aureus* species. The South African *C. officinalis* herbal extract may be considered as a possible alternative treatment for resistant pathogens.

**Limitations and recommendations**: This pilot study had some limitations. Regarding data collection, the concentration-dependent effects of different HE concentrations were not evaluated. Therefore, special attention should be paid to test the concentration-dependent characteristics of *C. officinalis* in different ethanol concentrations to gain in-depth information about the inhibitory activity of *C. officinalis*. Only one method (Kirby-Bauer disc diffusion method) was used to collect data. The study focused on the inhibitory effect and did not statistically analyze the results using relevant software. There is limited research on the antimicrobial activities of herbal extracts (HEs) and homeopathic mother tinctures (MTs). Therefore, this study serves as a baseline for further studies to evaluate the potential of medicinal plants as antibiotics.

## Conclusion

The rapid spread of antibiotic resistance necessitates the search for plant-based antibacterials. Due to their wealth in phytochemicals, medicinal plants provide a rich resource for producing novel antibacterial drugs. The current study attempted to demonstrate the inhibitory activities of ethanol herbal extract (HEs) and homeopathic mother tincture (MT) of *C. officinalis* flowers against ESKAPE pathogens and *E. coli* species.

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**Author contributions:** T-T analyzed the data and wrote and reviewed the final manuscript.

**Ethical considerations:** This study was approved by the University of Johannesburg Ethics Committee (REC-01-05-2022) before the commencement of the research.

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**Consent to participate:** Not applicable.

**Conflicts of interests:** There is no conflict of interest.

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