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Auranofin and Baicalin Inhibit *Clostridioides difficile* Growth and Sporulation: An *In vitro* Study

Lamiaa A. Madkour [iD]

Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Egypt.

Abstract

Clostridioides difficile is a principal cause of hospital-acquired gastrointestinal infections, with sporulation and toxin production being key determinants in the disease pathogenesis. Although infections have been escalating and the complications can be life-threatening, the narrow pipeline of approved therapeutics has not witnessed an equivalent surge. With the unfolding of worrisome mutations and antimicrobial resistance, attention has been drawn to either discovering new therapeutics, or even better, repurposing already available ones. Consequently, this study was undertaken to assess the anti-clostridial activity of auranofin, an anti-rheumatic FDA-approved therapeutic; and baicalin, a natural flavone glycoside with reported anti-microbial potential. In comparison with vancomycin, the *in vitro* efficacy of auranofin and baicalin was tested against hypervirulent *C. difficile* (BAA-1870™). Broth suspensions were prepared with and without the three agents and anaerobically incubated. At 24- and 48-hours post-incubation, serial dilutions were prepared and inoculated onto agar plates. Viable cell counts and viable spore counts were then quantified. Meanwhile, toxin production was assessed via ELISA. At a concentration as low as 3 µg/mL, auranofin demonstrated a potent anti-clostridial activity. Both auranofin and baicalin exhibited a remarkable reduction in *C. difficile* viable cell counts (*P*-value 0.03 for each) and spore counts (*P*-values 0.023 and 0.045 respectively). While auranofin and baicalin proved to be non-inferior to vancomycin as inhibitors of *C. difficile* growth, both drugs proved to be superior to vancomycin in decreasing the spore counts 48-hours post inoculation. Additionally, auranofin markedly reduced *C. difficile* toxin production (*P*-value 0.021); a feature that was deficient in both baicalin and vancomycin. To enrich the currently limited repertoire of anti-clostridial drugs, further research is encouraging to compare between the *in vivo* efficacy of auranofin and that of baicalin. Both agents represent promising approaches that could address the unfulfilled needs in controlling *C. difficile* infection.

Keywords: Auranofin, Baicalin, *Clostridioides difficile*, Diarrhea, Spores, Toxin, Vancomycin

*Correspondence: m_l@kasralainy.edu.eg

INTRODUCTION

Clostridioides difficile infection (CDI) constitutes the most serious etiology of antibiotic-associated diarrhea, which can eventually invoke a fatal outcome if improperly treated. Meanwhile, the treatment of CDI is an arduous challenge; with infections being mostly hospital-acquired, typically in the elderly patients who are infection-vulnerable due to comorbid conditions.[1,2] An additional challenge for treatment is the emergence of hypervirulent strains e.g. the PCR-ribotype 027 and the North American pulsotype 1 (NAP 1) that produce higher amounts of toxin A and toxin B; the main virulence factors of *C. difficile*. Furthermore, *C. difficile* spores are highly resistant to adverse conditions. Being able to survive in the patients' intestines until antibiotic cessation, these spores act as springboards that elicit relapses of infection.[3]

Even with adequate therapy, the recurrence rates of CDI are high. Within just a month of completing an antibiotic course for an initial episode, almost 15 - 30% of the patients will experience a recurrence and, of these, up to 60% will sustain further relapses.[4,5] In addition to being debilitating and undermining the patients' life quality, such relapses pertain to higher mortalities and uprising health care expenditures.[6,7]

The commonly employed drugs for treatment of CDIs include vancomycin and fidaxomicin, the former being toxic and the latter being overly expensive (150 times the cost of metronidazole).[7] The challenge of treatment is further compounded by the worldwide emanation of *C. difficile* resistance to these drugs.[8] Altogether, such challenges imperatively call for state-of-the-art anti-clostridial therapy that provides durable treatment outcomes.

Nonetheless, de novo drug discovery is a tedious process; where the development of a single new drug could take several years and cost billions. This has consequently directed researchers toward exploring alternative avenues for the treatment of CDI.

On the one hand, drug repurposing approaches have been gaining applause in various disease conditions. In this context, auranofin, an FDA-approved gold-containing anti-rheumatic drug has been investigated in the treatment of infections caused by *Staphylococcus aureus*, as well as by vancomycin-resistant enterococci.[7] In addition to being a potential anti-cancer agent, auranofin has been identified in some recent research as having anti-CDI activity. Possessing a thiol ligand with a high affinity to selenium, auranofin exhibits potency against selenium-consuming microorganisms such as *C. difficile*. Displacing the sulfur with selenium in that thiol ligand of auranofin deprives *C. difficile* from a key micronutrient. It has also been reported that auranofin inhibits redox enzymes which are indispensable for various cellular processes.[9] Meanwhile, previous reports have revealed that auranofin suppresses toxin production in several pathogens, such as *Staphylococcus aureus*, *Corynebacterium diphtheriae, Clostridium tetani, Clostridium botulinum,* and most recently, *C. difficile*.[10-13]

On the other hand, the overwhelming global drug resistance has revived the interest in herbal extracts for the treatment of a range of microbial infections. Some reports have shown that the multi-purpose herbs, *Scutellaria baicalensis,* displays a powerful *in vitro* antimicrobial action.[14] Baicalin, a flavone glycoside found in *Scutellaria baicalensis* has been employed in traditional medicine for the treatment of dysentery, hepatitis, pneumonia, and scarlet fever.[15] Moreover, earlier research has demonstrated an inhibitory effect of baicalin against *Staphylococcus saprophyticus*,[16] *Helicobacter pylori*,[17] Gram-negative pathogens,[14] and *Mycobacterium tuberculosis*.[18] The antibacterial activity of baicalin is postulated to involve the disruption of bacterial cell membranes; as well as inhibiting bacterial DNA, RNA, and protein synthesis.[19]

As a step in the pathway of probing novel anti-clostridial agents, the objective of this study was to compare between the *in vitro* antibacterial and antivirulence potential of each of auranofin and baicalin with relevance to the standard-of-care drug, vancomycin, against *C. difficile*.

MATERIALS AND METHODS

Bacterial Strain

Clostridioides difficile (reference

number: ATCC® BAA-1870™) was procured from Microbiologics (Minnesota, USA) representing an epidemic strain with increased sporulation. The strain is of ribotype 027, toxinotype IIIb, and has been identified by PCR as *tcdA*-positive, *tcdB*-positive and *cdtB*-positive. The strain was grown on brain heart infusion (BHI) agar (Himedia, Mumbai, India) at 37°C in anaerobic conditions (Figure 1).

Antimicrobial Agents

Auranofin, baicalin, and vancomycin powders (Figure 2) were obtained from Cayman Europe (Tallinn, Estonia). Auranofin and baicalin were dissolved in dimethyl sulfoxide (DMSO), while vancomycin was reconstituted in saline. All drugs were preserved as stock solutions.

Determining the Minimum Inhibitory Concentrations (MICs) of the Employed Agents

The broth microdilution assay was employed.[20] *C. difficile* broth suspension that was equivalent to 0.5 McFarland was added to the microplate wells containing auranofin, baicalin, and vancomycin at serial dilutions. Following anaerobic incubation for 24 hours, MICs were recorded as the minimum concentration of each agent that visually halted *C. difficile* growth.

Quantitation of Viable Bacterial Cells

Overnight *C. difficile* colonies were suspended in brain heart infusion (BHI) broth to yield a concentration of 0.5 McFarland.
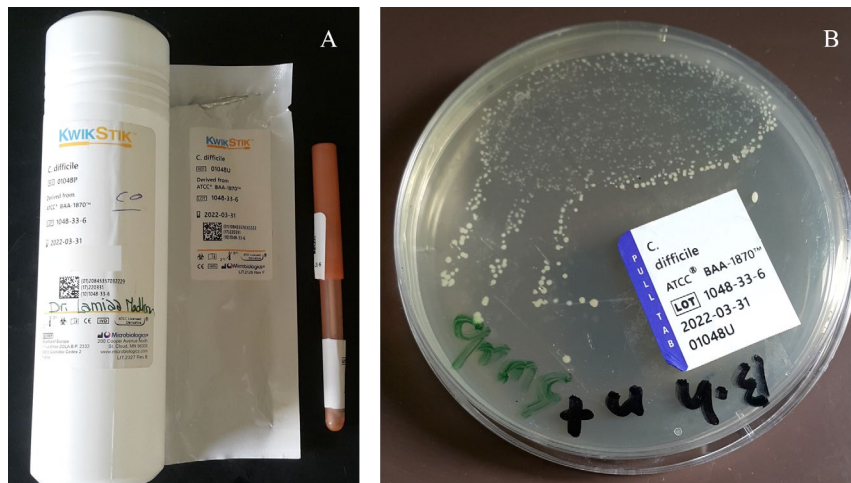
Figure 1. A) KWIK STIK device that contains lyophilized *Clostridioides difficile* pellet, an ampoule of hydrating fluid, and an inoculating swab. B) Early growth of *C. difficile* on brain heart infusion (BHI) agar supplemented with 0.1% Na⁺ taurocholate

Figure 2. Auranofin powder (25 mg), baicalin powder (250 mg), and vancomycin powder (250 mg)

Each of auranofin, baicalin, and vancomycin was added to the broth suspension at its reported MIC. Following anaerobic incubation of the broth with and without the drugs, serial dilutions (10^{-2} and 10^{-3}) were prepared at 24 and 48 hours.

From each dilution (neat, 10^{-2} and 10^{-3}), 100 μL were spread onto BHI agar plates. After anaerobic incubation for 24 hours, the average colony forming unit (CFU) per mL was calculated for each plate.[9,13]

ELISA

ELISA RIDASCREEN kit procured from R-Biopharm (Darmstadt, Germany) was employed. In addition to a control tube, auranofin, baicalin and vancomycin were added at subinhibitory concentrations (concentrations below the MIC) to *C. difficile* broth cultures which were then incubated in anaerobic environment. After 48 hours, the broth cultures were centrifuged at 10,000 rpm for five minutes, and the supernatant was used to perform the assay as per the manufacturer's booklet. The optical density corresponding to the total toxins concentration was measured at 450 nm wavelength and a reference filter of 630 nm, and compared for the three antimicrobial agents.[21]

Quantitation of Viable Bacterial Spores

In addition to a control tube, drugs at subinhibitory concentrations were added to *C. difficile* broth cultures, which were then incubated in an anaerobic environment at 37°C.[13]

At 24 and 48 hours post-incubation, a plate was cultivated from the control tube to determine the total count of vegetative cells plus spores. All tubes were then centrifuged twice, and in each time, the medium was replaced with phosphate buffered saline (PBS).[22] In order to destroy the vegetative cells, the tubes were then subjected to heat shock at 65°C for 45 minutes.

Dilutions were prepared from all tubes, and from each dilution, 100 μL were plated onto BHI agar supplemented with 0.1% sodium taurocholate (Sigma Aldrich, Missouri, USA); the latter being a potent inducer of spore germination. This was ensued by anaerobic incubation for 48 hours. The average colony forming unit (CFU) per mL was then calculated for each plate.
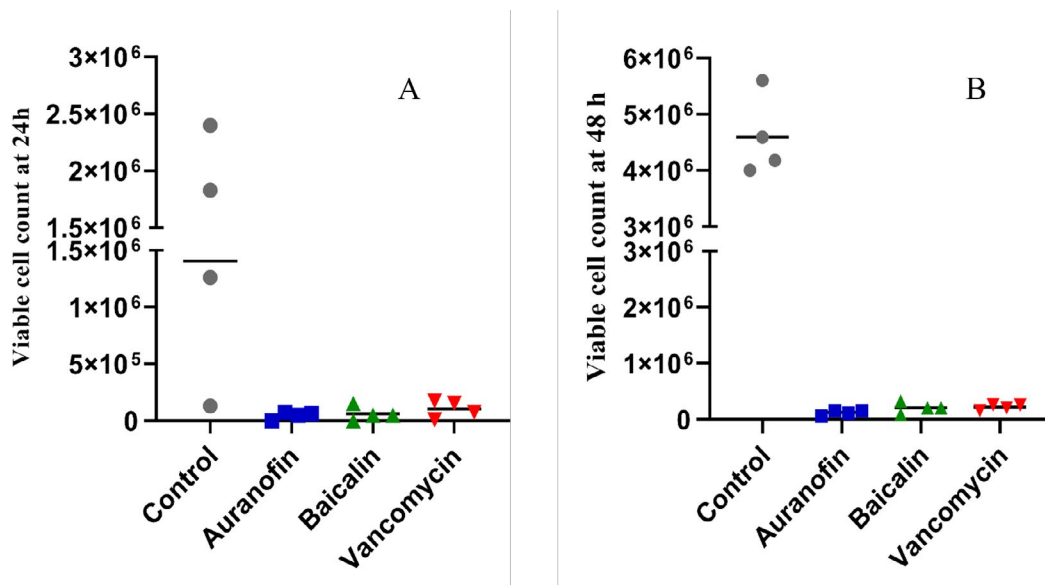
Figure 3. A) Number of viable vegetative cells from *Clostridioides difficile* cultures treated with auranofin, baicalin, or vancomycin assessed at 24-hours post-inoculation. B) Number of viable vegetative cells from *Clostridioides difficile* cultures treated with auranofin, baicalin, or vancomycin assessed at 48-hours post-inoculation. Numbers represent the colony forming unit (CFU) per mL

Statistical Analysis

Analysis was performed via SPSS version 24 and Graph pad prism version 9. One-way ANOVA and Kruskal Wallis tests were employed to assess the difference between drugs; followed by pairwise comparison by Bonferroni and Dunn test as appropriate. All of the *P*-values were two-sided. *P*-values ≤0.05 were regarded significant.

The relative change was defined as the difference between the final value and the initial value divided by the initial value, with one-fold change equivalent to 100% change.

All endeavors were replicated for at least three times.

RESULTS

Minimum Inhibitory Concentrations

The minimum inhibitory concentration of vancomycin against hypervirulent *C. difficile* ATCC BAA-1870 (ribotype 027) was revealed to be 2 µg/mL, while that of auranofin was 3 µg/mL, and that of baicalin was 800 µg/mL (0.08%).

Effect of the Tested Agents on Viable Cell Count

Each of auranofin and baicalin exhibited a pronounced reduction of *C. difficile* cell count following 24-hour incubation (*P*-values 0.031 and 0.033 respectively), as well as after 48-hour incubation (*P*-value <0.001 for each). These results were quite close to the 24 and 48-hour performance of vancomycin (*P*-values 0.037 and <0.001 respectively).

After 24-hour incubation, auranofin-treated culture demonstrated 0.97 fold decrease in *C. difficile* cell count, while baicalin-treated culture demonstrated 0.95 fold decrease, and vancomycin-treated culture demonstrated 0.93 fold decrease (Figure 3A).

After 48-hour incubation, auranofin-treated culture demonstrated 0.91 fold decrease in the cell count, while baicalin-treated culture demonstrated 0.85 fold decrease, and vancomycin-treated culture demonstrated 0.84 fold decrease (Figure 3B).

In comparison to vancomycin, auranofin exhibited a pronounced reduction in viable cell count 48-hours post-inoculation (0.026), but not after 24 hours (0.215). On the other hand, baicalin did not exhibit a significant reduction compared to vancomycin, either after 24 hours or after 48 hours.

Meanwhile, no difference was noticeable between auranofin and baicalin in reducing *C.*
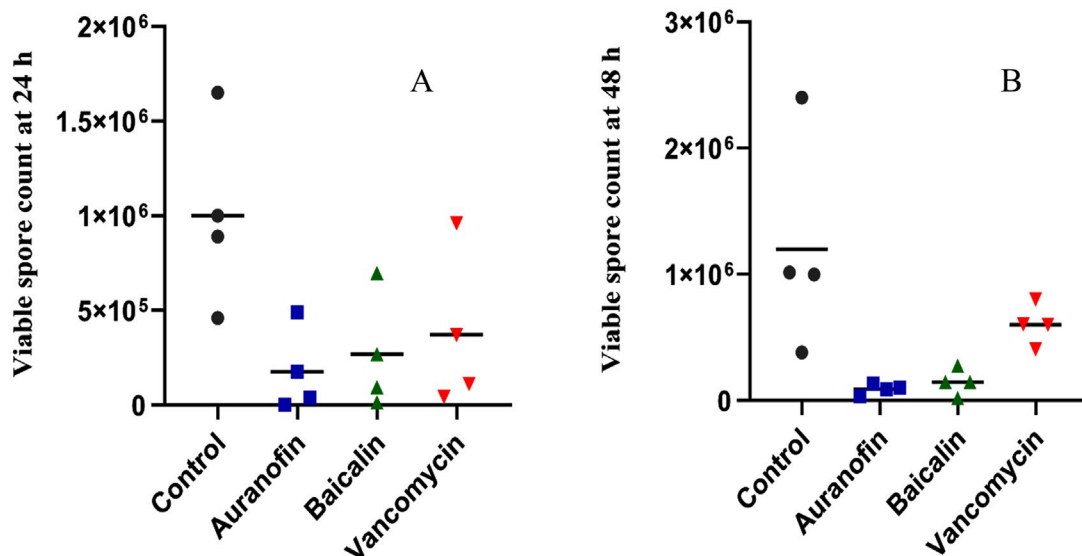
Figure 4. A) Number of viable spores from *Clostridioides difficile* cultures treated with auranofin, baicalin, or vancomycin assessed at 24-hours post-inoculation. B) Number of viable spores from *Clostridioides difficile* cultures treated with auranofin, baicalin, or vancomycin assessed at 48-hours post-inoculation. Numbers represent the colony forming unit (CFU) per mL.

difficile viable cell count, either after 24 hours or after 48 hours.

Effect of the Tested Agents on Viable Spore Count

Both auranofin and baicalin markedly reduced *C. difficile* viable spore count after 24-hour incubation (*P*-values 0.023 and 0.045 respectively), as well as after 48-hour incubation (*P*-values 0.041 and 0.05 respectively). On the other hand, vancomycin could not demonstrate a significant reduction of viable spore count either after 24 hours or after 48 hours (*P*-values 0.099 and 0.219 respectively).

After 24-hour incubation, auranofin-treated culture demonstrated 0.82 fold decrease in the spore count, while baicalin-treated culture demonstrated 0.73 fold decrease, and vancomycin-treated culture caused 0.63 fold decrease (Figure 4A).

After 48-hour incubation, auranofin-treated culture demonstrated 12.31 fold decrease in the spore count, while baicalin-treated culture demonstrated 11.68 fold decrease, and vancomycin-treated culture demonstrated 6.63 fold decrease (Figure 4B).

In comparison to vancomycin, both auranofin and baicalin exhibited a pronounced reduction in viable spore count 48-hours post inoculation (*P*-values 0.001 and 0.003 respectively), but not after 24 hours.

Meanwhile, no difference was noticeable between auranofin and baicalin in reducing *C. difficile* viable spore count, either after 24 hours or after 48 hours.

Effect of the Tested Agents on Toxin Production

Auranofin demonstrated a striking reduction in the total toxin production (toxins A and B) by *C. difficile* in comparison to the untreated control (*P*-value 0.021). In contrast, neither baicalin nor vancomycin could reduce the total amount of toxin produced (*P*-values 0.76 and 0.94 respectively). Of note, the amount of toxin was slightly elevated in the presence of baicalin (Figure 5).

In comparison to baicalin and vancomycin, it was noticeable that auranofin resulted in a remarkable reduction in total toxin production (*P*-values 0.009 and 0.026 respectively).
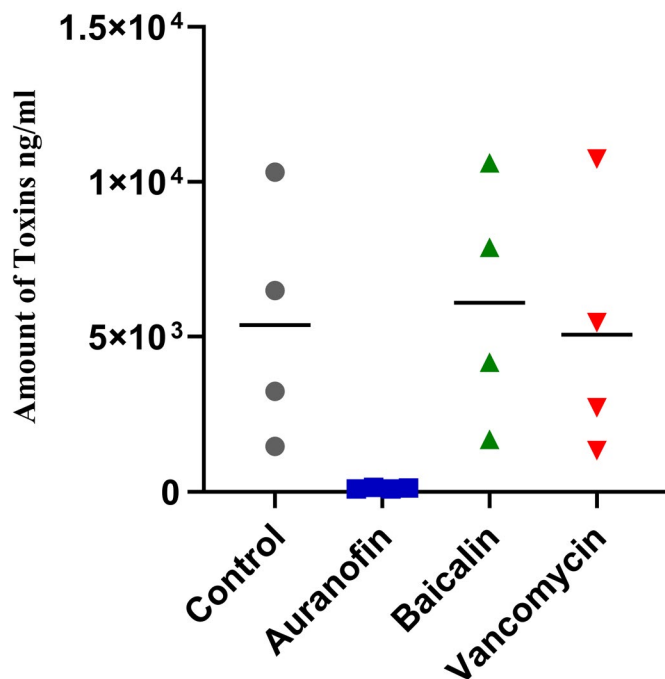
Figure 5. Effects of auranofin, baicalin, and vancomycin on *C. difficile* ATCC BAA 1870 total toxin production

DISCUSSION

Clostridioides difficile infection (CDI) has been enlisted by the CDC as one of the five urgent threats that mandate aggressive measures. Despite this fact, drug development against CDI has been proceeding at a much slower pace than coveted.[23]

Only three drugs are in current use; metronidazole and vancomycin, both discovered in the 1950s, and fidaxomicin, which gained approval in 2011. Nonetheless, the use of these drugs is hindered by a number of limitations. For instance, the estimated treatment failure rates are 14% and 22% for vancomycin and metronidazole, respectively. Additionally, 25-30% of those treated with either drug will experience recurrence.[24] Regrettably, the therapeutic outcome is still unsatisfactory even after fidaxomicin discovery.[25] Altogether, this underpins the compelling need for avant-garde effective therapeutics against CDI.

The discovery of novel antibacterial agents is a very lengthy and costly process. Meanwhile, the repositioning of an FDA-approved agent for a novel indication is an encouraging strategy for drug discovery. Owing to a meticulous clinical investigation, the pharmacodynamics, pharmacokinetics, and safety profile of such drugs have been well-investigated, which will unarguably lessen both the time and expenses pertinent to drug discovery.[26,27] Auranofin is an FDA-approved anti-rheumatic drug with a well-studied safety profile and limited adverse effects.[28] Owing to its poor absorption via the intestinal tract, auranofin possesses ideal pharmacokinetics as a therapeutic against CDI. With only 15-25% absorbability, almost 85% of the administered dose would reach the distal colon to be excreted in feces,[29] implying that auranofin can reach therapeutic concentrations at the infection site.[29,30]

On the other hand, antivirulence agents are currently gaining a foothold in halting resistant pathogens. Such therapeutic modality attenuates the pathogen's virulence rather than its growth.[31] Consequently, the pathogen's tendency toward developing resistance would be diminished; and moreover, the possibility of perturbing the beneficial microflora would be minimal.[21]

Baicalin is a plant-derived compound that has long been used in the treatment of various bacterial and viral infections.[15] In earlier research, it has been demonstrated as an inhibitor of bacterial protein synthesis, quorum sensing, and the expression of virulence genes.[32]

This study has been endeavored to assess the *in vitro* antibacterial and antivirulence activity of each of auranofin and baicalin against a hypervirulent *C. difficile* strain (ATCC® BAA-1870™), in comparison to the standard therapeutic agent, vancomycin.

In concordance with previous studies, auranofin was found to have a low MIC of 3 µg/mL against *C. difficile*; a concentration comparable to that of vancomycin MIC which was recorded as 2 µg/mL. These findings were consistent with those of Roder and Athan[9] who reported auranofin MIC as 4 µg/mL, as well as with those of AbdelKhalek et al.[13] who reported auranofin MIC range against *C. difficile* as 0.25-4 µg/mL and vancomycin MIC range as being 0.25-2 µg/mL. Meanwhile, Hutton et al.[18] reported auranofin MIC as 1.7 µg/mL, while another study revealed that both auranofin and vancomycin had the same MIC of 1 µg/mL against *C. difficile*.[33] On the other hand, baicalin MIC was found to be 800 µg/mL (0.08%). As previously demonstrated by Zhao et al.,[14] baicalin had an MIC of 4000 µg/mL against *E. coli*, while Wang et al.[16] reported baicalin MIC against *Staphylococcus saprophyticus* as 500 µg/mL.

In this study, each of auranofin and baicalin exhibited a pronounced reduction in the viable cell count of hypervirulent *C. difficile* (*P*-values 0.03 for each). This finding corroborates those of earlier research,[9,13] which revealed the superiority of auranofin in inhibiting *C. difficile* growth.

Meanwhile, *C. difficile* ability to sporulate is a fundamental virulence component in the disease process. Hypervirulent *C. difficile* possesses a higher ability to form spores that tolerate the standard disinfection procedures. This rationalizes the tendency of these strains to spread more readily in the environment. Following the conclusion of a treatment course, such spores can germinate in the intestine resulting in relapses.[34,35]

Remarkably, each of auranofin and baicalin resulted in a noticeable inhibition of *C. difficile* sporulation (*P*-values 0.023 and 0.045 respectively). These findings reiterate those of Roder and Athan,[9] and AbdelKhalek et al.[13] who

reported an anti-spore activity of auranofin, as well those of Pellissery et al.[21] who demonstrated an anti-spore activity of baicalin.

It has been demonstrated that auranofin disrupts several protein synthesis pathways in *Staphylococcus aureus*. Baicalin has also been reported as an inhibitor of bacterial DNA and protein biosynthesis.[19] Hence, it can be conjectured that auranofin and baicalin inhibit the synthesis of the coat proteins in *C. difficile* spores.[36]

Noteworthy, the ability of auranofin and baicalin to suppress sporulation would be reflected as improved treatment outcomes, lower rates of CDI relapses, and reduced infection transmission.

In the meantime, vancomycin caused a much less reduction of *C. difficile* sporulation. This was consistent with the observation reported by Baines et al.[37] and AbdelKhalek et al.[13] who detected almost no reduction in the spore count with vancomycin. Contradicting this finding, Roder and Athan[9] reported an ability of vancomycin to reduce *C. difficile* spore count. Of note, the response to vancomycin can vary in different strains; while *C. difficile* BAA-1870 was used in this study and in the study by AbdelKhalek et al., Roder and Athan used *C. difficile* M7404.

On the other hand, toxins A and B are pivotal for *C. difficile* to provoke a disease. Furthermore, toxin production is augmented by environmental stresses; consequently, some anti-clostridial agents (e.g. vancomycin) could promote *C. difficile* toxin secretion.[38]

Hence, an additional approach in this study was to assess the activity of the three drugs at subinhibitory concentrations against toxin production by *C. difficile*.

In concordance with earlier studies,[9,13] auranofin exerted a substantial inhibition of toxin production by *C. difficile*. Nonetheless, baicalin at a subinhibitory concentration did not significantly affect toxin production. This was in contrast to a study by Pellissery et al.[21] who reported 70-85 % reduction in *C. difficile* toxin concentration in the presence of baicalin. Corroborating earlier studies,[13,39] vancomycin could not reduce the amount of toxins produced by *C. difficile* in the present study.

Further *in vitro* research is justified to elucidate the mechanisms by which auranofin and baicalin interfere with *C. difficile* sporulation. It is also rationalized to evaluate the effect of baicalin on toxin production in a wider panel of *C. difficile* strains. Another fundamental factor that ought to be investigated is the impact of each of auranofin and baicalin on the gut microbiome; where a possibility of dysbiosis could elevate the risk of CDI recurrence.[40]

CONCLUSION

Auranofin demonstrated a substantial reduction in *C. difficile* viable cell count as well as viable spore count. Additionally, auranofin markedly reduced the amount of toxin produced by *C. difficile*.

Meanwhile, baicalin proved to be non-inferior to vancomycin in inhibiting *C. difficile* growth and spore formation; but at subinhibitory concentration, it could not reduce toxin production.

Although auranofin proved superior to baicalin in reducing toxin secretion, the possibility of auranofin-induced resistance should be thoroughly investigated. Collectively, these results underpin that auranofin and baicalin represent potential safe and effective additions to the current pipeline of CDI treatment. Nonetheless, follow-up *in vivo* investigations are warranted for dose standardization and long-term safety. This can be ensued by clinical trials in human subjects.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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