Screening of novel synthetic agents against *Clostridioides difficile*

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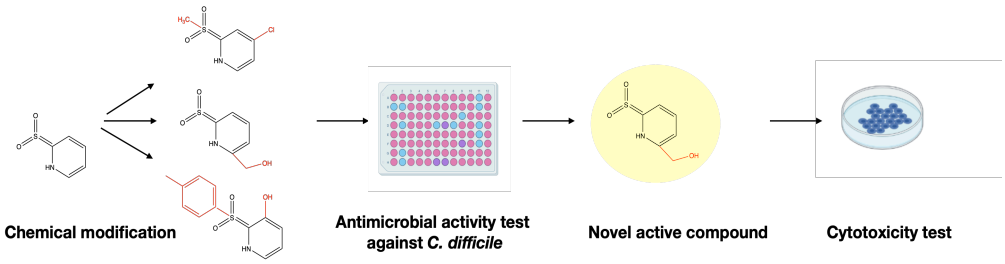
Abstract: Antibiotic resistance (AR) is the biggest concern in public health that causes tremendous economic loss and fatalities. *Clostridioides difficile* is the most common cause of hospital-acquired diarrhea, with symptoms ranging from mild diarrhea to life-threatening colitis. On top of that, the pathogen is being known to resist multiple antibiotics. Due to the high background of AR, reduced susceptibility to prescribed antibiotics have been continuously reported in this pathogen. Chemical synthesis allows us to access to the first antimicrobial agents before the discovery of penicillin, and the process also enables the development of drug with the desired characteristics and higher efficiency. This study integrated the science of chemistry together with biology to seek novel antimicrobial agents against *C. difficile*. A series of compounds were chemically synthesized from five different core structures; (i) 3-methyl-5-pyrazolones, (ii) 2-sulfonylquinolines, (iii) 2-sulfonylpyridines, (iv) sulfoximines, (v) pentacyclic triterpene as they were reported to link with biological activities. A total of 77 compounds of 5 different groups were tested for antimicrobial activity against *C. difficile* strain R20291 using broth microdilution method. None of the chemical synthetic compounds in group i to iv exhibited inhibition concentration of lower than 10 μM against *C. difficile* R20291, while 2 semisynthetic compounds, AM1 and AM2 from the group v could inhibit *C. difficile* R20291 with MIC of 5-10 μM. Interestingly, all pentacyclic triterpene derivatives exhibited lower cytotoxicity to HaCaT cells compared to the parental compound.

Graphical abstract:



Keywords: antibiotic resistance; *Clostridioides difficile;* medical chemistry

1. Introduction

Antibiotic resistance (AR) was recognized as one of the biggest threats to global health and sustainability as 700,000 patients die each year form AR infection and the number seem to be rising [1]. AR refers to the ability of bacteria to become resistant to antibiotics designed to kill them, in other words, used antibiotics are no longer effective to cure disease. Because AR occurs everywhere, it can cause a domino effect resulting to a chain of problems starting from difficulty of medical treatment and could end up with the economic collapse.

Clostridioides difficile is best known to have high AR background [2]. Not just only a single drug, but *C. difficile* resists to wide range of antibiotics, including those with broad-spectrum activities [3]. So, patients with *C. difficile* infection (CDI) usually confront with recurrence, complicated treatment and high risk of mortality. The main reason that drives bacteria to show high durability is having diverse AR mechanism [3]. Antibiotic is considered to be the most important risk factor for CDI. It has been estimated that 10% of patients receiving antibiotics are suffered from antibiotic-associated diarrhea, of which 20% are reported to be CDI [4]. Incidence and severity of CDI has been dramatically increased in the hospital worldwide. Over the past 20 years, several outbreaks of CDI were reported across the world, including America, Europe, Asia and Oceania owing to the emergence of hypervirulent drug-resistant strains which are highly resistant to several antibiotics, especially on a group of fluoroquinolone [5–7]. For CDI treatment, metronidazole and vancomycin are commonly prescribed as the first- and second-line antibiotics, respectively. Unfortunately, emergences of *C. difficile* with less susceptibility to those therapeutic drugs had been evidenced as well as clinical reports regarding recurrence [8–10]. Thus, novel antimicrobial agents are urgently needed for combating with CDI and emergence of antibiotic resistance.

Medical chemistry plays an important role toward drug discovery which involves in compound selection, design, and chemical synthesis. In the 1910s, salvarsan, the first antimicrobial agent was chemically synthesized by Paul Ehrlich for treating syphilis, a disease that had haunted European country for several centuries. The substance was called "Magic Bullet, since it could eliminate bacteria without harmful effects on human health [11]. Consequently, chemical synthesis allows the discovery of several drugs, which contain activities against bacteria, fungi, and even cancer [12–15]. Two types of compounds can be derived from the process of medical chemistry based on the source of the original compound; synthetic and semi-synthetic agents. The synthetic drug is an artificial compound derived from a fully chemical synthetic process which is not involved in natural precursors. Fluoroquinolones, oxazolidinones, and sulfonamides are well-known antimicrobial agents that belong to synthetic drug [16,17]. Semisynthetic agent is a partial compound modification in which the compound derived from natural sources are modified to enhance its activity. Semisynthetic drugs are usually designed to have low toxicity to human [18], meanwhile, their properties are enhanced in terms of compatibility, solubility, and efficiency [19,20].

This study aims to screen for novel antimicrobial agents against *C. difficile* from the compound library composed of synthetic agents, which have the potential to possess pharmaceutical activity together with semi-synthetic agents of natural product with pentacyclic triterpene core; asiatic acid. As our previous study revealed the inhibitory effect of asiatic acid, an active agent found in *Centella asiatica,* on several strains of *C. difficile* [21].

2. Materials and Methods

2.1 Bacterial strain and culture condition

C. difficile R20291 was initially cultured on cycloserine-cefoxitin fructose agar (CCFA) as a selective medium for *C. difficile.* Following cultivation and maintenance of the bacterium were proceeded in Brain Heart Infusion (BHI) medium.

2.2 Chemical agents

Five core structure compounds; 3-methyl-5-pyrazolones, 2-sulfonylquinolines, 2-sulfonylpyridines, sulfoximines, and pentacyclic triterpene were used as an initiating lead compound to generate synthetic agents. Asiatic acid with 90% purity (Hunan Sunshine Bio-Tech Co., Ltd., China) was purchased for semi-synthesizing of derivative compounds.

2.3 Screening of antimicrobial agents

Broth microdilution method was performed in 96-well plate to investigate the antimicrobial activity of both synthetic and semi-synthetic compounds. All of the compounds were prepared in DMSO. A single colony of *C. difficile* R20291 from CCFA plate was preculture overnight in BHI broth, followed by subculturing in a new BHI broth for 6 h to avoid bacterium entering spore stage under anaerobic condition. Afterward, the turbidity of bacterial suspensions was adjusted to 0.5 McFarland and further dilute in BHI broth with the ratio of 1:15 (culture: media) according to the standard protocol [22]. Ten µl of the diluted bacterial suspension was seeded into a microplate pre-filled with 100 µl of BHI with 10 µM of synthetic or semi-synthetic compounds. After 2 days of incubation at 37°C under anaerobic condition, growth visibility of the bacterium was determined by monitoring OD_{600} using a microplate reader (Tecan). The compound with inhibition concentration at 10 µM is designated to have antimicrobial activity against *C. difficile*.

2.4 Cell culture and Cell viability assay

HaCaT, an immortalized human keratinocyte cell line, was used for cytotoxicity test. HaCaT was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2 humidified incubator. To test the cell cytotoxicity of the compounds on HaCaT, a cell viability assay was performed. HaCaT cells were treated with different concentrations of compounds including 0, 1, 10, and 50 µM for 48 hours. Cell viability was obtained using Cell Counting Kit-8 (CCK-8).

3. Results

3.1 Synthetic and semi-synthetic agents

A total of 77 compounds were tested for anti-clostridial in this study. Seventy-four compounds were chemically synthesized from 4 different lead compounds, while 3 different compounds namely, AM1, AM2, and AM3, were successfully semi-synthesized using a pentacyclic triterpene, asiatic acid, as a lead compound (Figure 1).

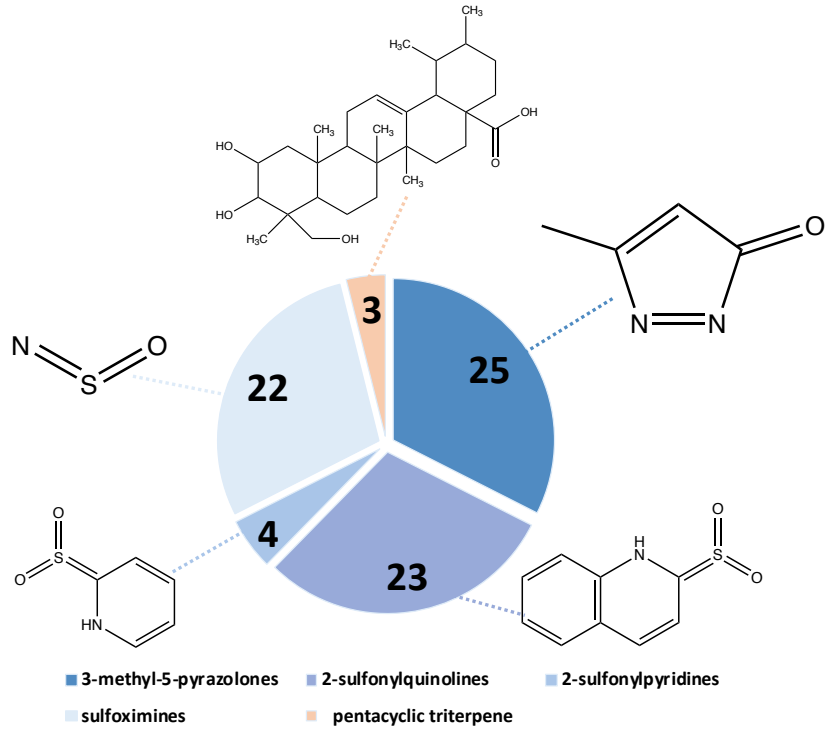


Figure 1. Synthetic and semisynthetic agents used for screening of inhibitory activity against *C. difficile.*

3.2 Screening of antimicrobial agent against C. difficile R20291

All of the 77 compounds were screened for an inhibitory activity against *C. difficile* R20291 through broth dilution method. Seventy-four synthetic compounds had inhibition concentration of greater than 10 μM toward *C. difficile* R20291. As the inhibition threshold of the inhibition was set to 10 μM, therefore, all synthetic compounds here in this study were assigned to have no inhibitory effect against *C. difficile*. For the three of semi-synthetic compounds derived from Asiatic acid (AM1, AM2, and AM3), two Asiatic acid derivatives, AM1 and AM2, exhibited inhibitory effect at 10 μM. Hence, AM1 and 2 were selected for determining minimum inhibitory concentration (MIC).

3.3 Minimum inhibitory concentration of the semisynthetic compounds

MIC of AM1, AM2 and AM3 were investigated against *C. difficile* R20291. As shown in table 1, AA, a natural compound, showed an MIC value of 10 μM against *C. difficile* R20291. AM1 and AM2 exhibited superior inhibitory effect against *C. difficile* R20291 with the MIC of 5 μM. Interestingly, the MIC of AM3 was raised to 20-40 μM which calculated to be up to 4 times higher than those of the parental compound.

Table 1. Antimicrobial activity of asiatic acid (AA) and AA derivatives (AM1, AM2, and AM3) against *C. difficile* R20291.

Compound	**MICs (μM)**[1]
	***C. difficile* R20291**
AA	10
AM1	5
AM2	5
AM3	20-40

[1] The MIC values were acquired from three independent experiments.

3.4 Cytotoxicity test against HaCaT cells

After the HaCaT cells were treated with compounds for 48 hours, the percentage of cell viability showed a significant decrease in a dose-dependent manner as shown in Figure 2. Considering the concentration of a compound at 1 μM, AA provided the most toxicity among the others with 72 % viability. Whereas, percent viability of HaCat cells treated with 1 μM of the compounds was recovered up to 95 %, 102%, and 83% in AM1, AM2, and AM3, respectively. At 50 μM percentage of HaCaT cells viability showed no difference in all tested compounds.

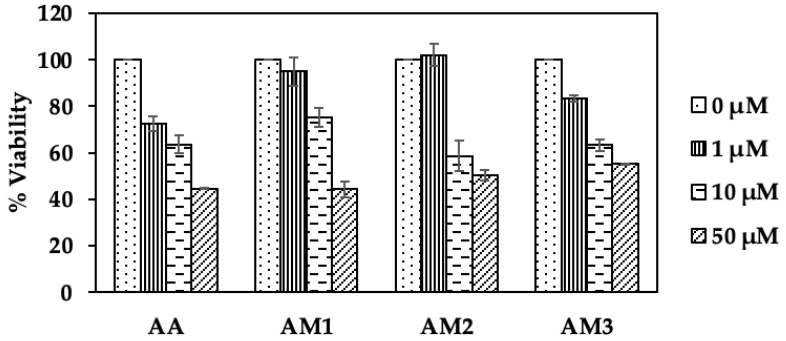


Figure 2. Effect of Asiatic acid and its derivatives on cell viability of HaCaT cells. Cell Counting Kit-8 was performed to obtain cell viability.

4. Discussion

The situation of antibiotic resistance was announced by world health organization (WHO) to be at dangerously high levels worldwide. Emergences and spreading of antibiotic resistance led to a complicated treatment or sometime treatment is impossible. As prescribed drugs become less effective, more of the money has to be invested to medical costs and patient has to stay at the hospital longer, which may result in a higher in the mortality rate. Therefore, novel agents, which are effective against antibiotic resistant bacteria, are urgently need for tackling the problem.

In this study, 74 synthetic agents synthesized from 4 different leading compounds as well as 3 Asiatic acid derivatives were screened for inhibitory effects against *C. difficile*, bacterium known to be resistant to multiple antibiotics. No anticlostridial activity was detected from synthetic agents derived from 3-methyl-5-pyrazolones, 2-sulfonylquinolines, 2-sulfonylpyridines, and sulfoximines at the concentration of 10 μM. Oxazolidinones, sulfoximine based antibiotic, are known to possess a broad range of antimicrobial activity with the range of MIC of 0.05-10 μg/mL [23]. Desai et al. reported the antimicrobial activity of three quinoline derivatives against *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli,* and *Pseudomonas aeruginosa* with the range of 12.5-50 μg/ml [24]. While the quinoline derivative synthesized by Fu et al. showed to be an effective antimicrobial agent with MIC of 0.125-8 μg/ml [25]. The report of antimicrobial activity of sulfonylpyridine derivatives was addressed against *S. aureus* and *B. substilis*; however the inhibition concentrations were observed to drastically high (0.16-3.36 μM) [26]. Although, several pyrazolone derivatives were reported to possess antimicrobial activities with the MIC ranging from 0.5-250 μg/ml against various pathogenic bacteria, no inhibitory activity against *C. difficile* was mentioned [27–30]. No anticlostridial activity found in all of the synthetic agents could be due to the inhibition concentration of the compounds that may be higher than the threshold line, 10 μM. Modification sites and added chemical groups may not be suitable for promoting the antimicrobial activity of the compounds. It should be noted that *C. difficile* is capable of resisting several antibiotics. Therefore, a series of synthetic compounds may somehow being eliminate from the bacterium through mechanism of resistances harbor by *C. difficile*.

Asiatic acid is a pentacyclic triterpenoid that is usually found as plant essential oils, particularly in *Centella asiatica*. Asiatic acid had been previously reported to possess

antimicrobial activity against several foodborne pathogens such as *Escherichia coli, Salmonella* Typhimurium, *Pseudomonas aeruginosa, Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus cereus* with the MIC of 20-40 μg/ml [31]. Additionally, Asiatic acid was also reported to inhibit the growth of several isolates of *C. difficile* with the MIC of 20-40 μM potentially through the membrane disruption mechanism [21].

Several chemical modifications on Asiatic acid had been evident for improving pharmacological activity. Huang et al. reported that Asiatic acid derivatives containing α-aminophosphonate exhibited higher level of antitumor activities compared to those of the original compound [32]. An increase in anti-tumor was also reported in the novel AA derivatives by Jing et al. [33]. Despite numbers of AA were designed and chemically synthesized to improve the pharmacological activities, most of the attempts were focusing on anti-cancer, neurodegenerative diseases, antidiabetic activity, and hepatoprotective activity [34]. No report on modification of Asiatic acid on the prospective of antimicrobial agent has been addressed so far. Here, we reported AM1 and AM2, two Asiatic derivatives, which exhibited an improvement of the anti-clostridial activity up to 2-fold compared to the parental compound with less toxicity to the cell line. It is possible that functional groups that were added to the modification site of AM1 and AM2 may allow better interaction between the agents and the target. Another possibility is the higher water-solubility resulting from the medication as higher water-solubility was reported to increase antimicrobial activity of chitosan [35,36]. Additionally, reduction of cytotoxicity observed in Asiatic derivatives, particularly AM1 and AM2 at 1 μM. This study pointed out the benefit of medical chemistry not only for improving the antibacterial activity of biological compounds but also reducing their cytotoxicity for better therapeutic outcomes.

Author Contributions:

PH: Conceptualization, Methodology, Writing - Original Draft, Supervision. **MP**: Methodology, Validation, Investigation. **MY**: Validation, Investigation. **NP:** Validation, Investigation. **PP**: Supervision. **SY**: Resources. **TJ**: Conceptualization, Methodology, Writing - Review & Editing, Supervision.

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Conflicts of Interest:

The authors declare no conflict of interest

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