Research Journal of Pharmacognosy (RJP) 9(4), 2022: 21–27
Received: 28 Oct 2021
Final revision: 2 July 2022
Accepted: 17 July 2022
Published online: 26 July 2022
DOI: 10.22127/RJP.2022.349694.1935

Original article

Antibacterial and Antifungal Activities of Andrographolide in Combination with Antimicrobial Drugs

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Abstract

Background and objectives: The resistance of infectious pathogens to antimicrobial drugs is an underestimated threat to public health. This rapidly developing phenomenon necessitates the discovery of new treatment strategies. Combining natural compounds with first-line antimicrobials is one treatment strategy to mitigate the emergence of resistant pathogens. Andrographolide, a diterpene lactone isolated from *Andrographis paniculata* has been reported to possess potent anti-infective activity. This study was conducted to evaluate the combination effect of andrographolide with first-line antimicrobial drugs to fight emerging resistance. **Method**: The minimum inhibitory concentration (MIC), fold increase in antimicrobial efficacy and fractional inhibitory concentrations (FIC) of andrographolide and ceftriaxone, ciprofloxacin, amoxicillin, metronidazole, amikacin, clindamycin and fluconazole were determined using the high throughput spot culture growth inhibition (HT-SPOTi) assay against ten isolated clinical strains; *Streptococcus pyogenes, Escherichia coli, Vibrio cholerae, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumoniae, Enterococcus faecalis, Salmonella paratyphi* B and *Candida albicans.* **Results**: Combination of andrographolide and the first-line antimicrobials showed various degrees of susceptibility and efficacy against the tested microorganisms with the highest MIC, 0.85 µg/mL recorded. The FICI (Fractional Inhibitory Concentration Indices) for synergy ranged between 0.00 to 0.28 depending on the microorganism and antimicrobial drug. **Conclusion**: Use of andrographolide with first-line antimicrobials could aid in combating the menace of resistance pathogens. However, this should be done with caution as some of the antimicrobials tested exhibited antagonistic effects.

Keywords: andrographolide; antibacterial activity; antimicrobial drugs; high-throughput screen

Citation: Ativui S, Danquah CA, Ofori M, Gibbons S, Bhakta S, Doe P. Antibacterial and antifungal activity of andrographolide in combination with antimicrobial drugs. Res J Pharmacogn. 2022; 9(4): 21–27.

Introduction

Despite conscious efforts to reduce the burden of infectious diseases, emerging and re-emerging infections caused by pathogens, including bacteria and fungi remain public health threats in developing countries as a result of globalization [1]. Antimicrobial drugs were one of the most effective tools in the battle against infections and have substantially improved human health since

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their inception. Currently, these health advantages have been jeopardized since many frequently used antimicrobials have become less efficient against certain infections in humans and animals due to the development of resistance [2,3]. The increasing phenomenon of resistance is a concern, attributed to antimicrobial abuse and misuse. As a result, microorganisms have developed multiple mechanisms of resistance with no effective therapy [4].

One strategy that could be employed to overcome these resistance mechanisms is the use of combination therapy. Combination therapy involves the simultaneous administration of two or more drugs, in dosage forms that contain more than one active component. A combination can be made of different natural compounds or a natural compound combined with standard antimicrobial drugs. The rationale for combination therapy is to prevent the emergence of resistant strains and encourage the use of lower doses of drugs to minimize toxicity [5].

Andrographolide is a diterpene lactone, the predominant constituent of *Andrographis paniculata* (Burm. f.) Wall. ex Nees, a plant used in Asian traditional medicine to treat bacterial infections. Investigations on the antimicrobial activity of andrographolide demonstrated potent anti-infective properties [6]. Thus, this study conducted a preliminary investigation to evaluate the activity of andrographolide combined with first-line antimicrobials against infectious microorganisms as an alternative therapy to improve antimicrobial resistance. In addition, to the best of our knowledge, very little data exists on combining andrographolide with standard antimicrobial drugs using the high throughput spot culture growth inhibition (HT-SPOTi) method from our review of literature.

Materials and Methods

Ethical considerations

Ethics in research were considered during the study.

Chemicals and reagents

Andrographolide was purchased from Shanxi Chemical Research Institute (Wanbailin District, Shanxi, China). Antimicrobial powders of ceftriaxone, ciprofloxacin, amoxicillin, metronidazole, amikacin, clindamycin, Mueller-Hinton agar and phosphate buffered saline fluconazole were purchased from Sigma Aldrich (St. Louis, USA).

Bacterial strains

Ten isolated clinical strains; *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 25922, *Vibrio cholerae* ATCC 14035, *Pseudomonas aeruginosa* ATCC 27316, *Staphylococcus aureus* ATCC 6538, *Proteus mirabilis* ATCC 29906, *Klebsiella pneumoniae* ATCC 700603, *Enterococcus faecalis* ATCC 51299, *Salmonella paratyphi B* ATCC 9150 and *Candida albicans* ATCC 10231 were obtained from the Department of Microbiology, Faculty of Pharmacy and Pharmaceutical sciences, Kwame Nkrumah University of Science and Technology. Frozen stocks of the microorganisms were cultured on Mueller-Hinton agar and incubated at 37^{o} C for 24 h. Single colonies of each specie was suspended in 10 mL of phosphate buffer saline until a turbidity equivalent to 0.5 McFarland standard was achieved.

Anti-infective assay

All media was prepared according to standard laboratory protocol. The HT-SPOTi method was utilized in the anti-infective assay to determine the MICs of andrographolide and the antimicrobial drugs independently [7]. Microorganisms were sub-cultured from a cryopreserved glycerol stock and incubated at an optimum temperature for 24 h. Nutrient agar was autoclaved, placed in a water bath and the temperature kept between 60-65 ^{o}C. Antimicrobials were weighed, dissolved in an appropriate volume of 1% dimethyl sulfoxide (DMSO) to give a stock concentration of 50 mg/mL. A two-fold serial dilution of stock solution was carried out in a PCR half skirted plate with concentrations ranging from 50-1 mg/mL. Then 7 µL of the antimicrobials was transferred into the corresponding 96-well microplate with a multi-channel pipette, 200 µL of molten agar was dispensed to the wells. The plates were then swirled. A multi-channel pipette was used to dispense 7 µL of microbial suspension into the corresponding wells. The microbial suspension was allowed to diffuse into the agar for 5 minutes. The plates were sealed with parafilm, wrapped in aluminum foil, inverted and incubated at 37 ^{o}C. The MIC was recorded within 24 h.

Combination anti-infective assay

In the combination study, 25 mg of the antimicrobials and 25 mg of andrographolide were weighed, dissolved in an appropriate volume of 1% DMSO to achieve a stock concentration of 50 mg/mL. The method described above was repeated i.e., a combination of andrographolide with the antimicrobials was tested concurrently. The following parameters were evaluated to investigate if there was any impact on the efficacy of the antimicrobial and determine if there was either synergism or antagonism.

The percentage fold increase in Antimicrobial Efficacy was calculated as follows [7,8]:

$$\text{Fold increase in antimicrobial efficacy (\%)} = \frac{\text{MIC of antimicrobial} - \text{MIC of antimicrobial combined with Andrographolide}}{\text{MIC of antimicrobial}} \times 100$$

The Fractional Inhibitory Concentration Index (FIC) was calculated with the equation.

$$\text{FIC} = \frac{\text{MIC of antimicrobial in the presence of Andrographolide}}{\text{MIC of antimicrobial}} + \frac{\text{MIC of antimicrobial in the presence of Andrographolide}}{\text{MIC of Andrographolide}}$$

Synergism is indicated by a FIC value of less than 0.5, no interaction is shown by a FIC index between 0.5 and 4.0, and antagonism is indicated by a FIC index greater than 4.0 [7].

Results and Discussion

The assessment of anti-infective activity was based on the MIC, which was the lowest concentration of andrographolide and antimicrobial that showed no visible growth in the Mueller-Hinton agar after incubation. From the results (Table 1), the tested microorganisms exhibited varied susceptibility and resistance to andrographolide. Andrographolide showed bactericidal activity against *Streptococcus pyogenes, Klebsiella pneumoniae, Salmonella paratyphi* B and the highest inhibitory activity against *Vibrio cholerae* (1.71 µg/mL). *Staphylococcus aureus, Proteus mirabilis, Enterococcus faecalis and Candida albicans* were found to be resistant (875 µg/mL).

The highest MICs (Table 2), fold increase in antimicrobial efficacy (Figure 1) and FICI (Table 3) were obtained when andrographolide was combined with the antimicrobial drugs as follows: ceftriaxone against *Enterococcus faecalis* (<0.85 µg/mL, 99.9%, <0.03), *Vibrio cholerae* (<0.85 µg/mL, 99.9%, <0.13), and *Candida albicans* (<0.85 µg/mL, 99.8%, <0.00); ciprofloxacin against *Pseudomonas aeruginosa* (<0.855 µg/mL, 99.2%, <0.63), *Enterococcus faecalis* (<0.85 µg/mL, 99.9%, <0.01), *Vibrio cholerae* (<0.855 µg/mL, 87.5%, <0.13), *Salmonella paratyphi B* (<0.85 µg/mL, 50%, <0.63) and *Candida albicans* (<0.855 µg/mL, <0.06); amoxicillin against *Enterococcus faecalis* (109.38 µg/mL, 93.7%, 1.13); metronidazole against *Proteus mirabilis* (3.42 µg/mL, 99.8%, 0.01); amikacin against *Enterococcus faecalis* (<0.855 µg/mL, 99.9%, <0.01); clindamycin against *Proteus mirabilis* (1.71 µg/mL, 93.7%, 0.07); fluconazole against *Candida albicans* (1750 µg/mL, 0%, 257.85), respectively.

Bioactive constituents from plants are vital therapeutic compounds that aid the treatment of infectious diseases [9].

In this study, the anti-infective activity of andrographolide was determined with the high throughput spot culture growth inhibition (HT-SPOTi) method.

Table 1. The minimum inhibitory concentrations of andrographolide and antimicrobials against infectious pathogens

Drug	*S. aureus*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*E. faecalis*	*P. mirabilis*	*V. cholerae*	*S. pyogenes*	*S. paratyphi B*	*C. albicans*
Minimum inhibitory concentration (µg/mL)										
Andrographolide	875	109.38	109.38	6.84	875	875	1.71	6.84	6.84	875
Ceftriaxone	27.34	54.69	109.38	218.75	875	54.69	875	54.69	54.69	437.5
Ciprofloxacin	109.38	218.75	109.38	218.75	875	1.71	6.84	109.38	1.71	13.67
Amoxicillin	109.38	437.5	54.69	1750	875	1750	1750	218.75	6.84	NT
Metronidazole	>1750	54.69	54.69	27.34	>1750	>1750	1750	218.75	13.67	1750
Amikacin	218.75	109.38	109.38	437.5	1750	1.71	437.5	54.69	3.42	875
Clindamycin	437.5	218.75	27.34	218.75	1750	27.34	1750	54.69	6.84	437.5
Fluconazole	NT	NT	NT	NT	NT	NT	NT	NT	NT	6.84

NT: not tested; Microorganisms: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Proteus mirabilis, Vibrio cholerae, Streptococcus pyogenes, Salmonella paratyphi B, Candida albicans*

Table 2. The minimum inhibitory concentrations of andrographolide combined with antimicrobials against infectious pathogens

Andrographolide combined with antimicrobials	*S. aureus*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*E. faecalis*	*P. mirabilis*	*V. cholerae*	*S. pyogenes*	*S. paratyphi B*	*C. albicans*
Minimum inhibitory concentration (µg/mL)										
Ceftriaxone	1.71	437.5	218.75	27.34	<0.855	>1750	<0.855	6.84	3.42	<0.855
Ciprofloxacin	3.42	3.42	<0.855	54.69	<0.855	1.71	<0.855	13.67	<0.855	<0.855
Amoxicillin	218.75	>1750	>1750	218.75	109.38	>1750	218.75	1750	875	NT
Metronidazole	437.5	>1750	>1750	1750	875	3.42	1750	1750	109.38	1750
Amikacin	27.34	218.75	437.5	3.42	<0.855	1.71	54.69	109.38	1.71	109.38
Clindamycin	218.75	1750	1750	218.75	437.5	1.71	875	1750	437.5	437.5
Fluconazole	NT	NT	NT	NT	NT	NT	NT	NT	NT	1750

NT: not tested; Microorganisms: *Staphylococcus aureus Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Proteus mirabilis, Vibrio cholerae, Streptococcus pyogenes, Salmonella paratyphi B, Candida albicans*

Table 3. The fractional inhibitory concentration index of andrographolide combined with antimicrobials

Andrographolide combined with antimicrobials	*S. aureus*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*E. faecalis*	*P. mirabilis*	*V. cholerae*	*S. pyogenes*	*S. paratyphi B*	*C. albicans*
Fractional inhibitory concentration index										
Ceftriaxone	0.28	1	128.17	0.5	<0.03	>34	<0.13	0.016	0.56	<0.00
Ciprofloxacin	0.53	0.05	<0.63	1	<0.01	1	<0.13	0.03	<0.63	<0.06
Amoxicillin	32.98	>20	>1024.39	6	1.13	>3	32.11	4	255.85	NT
Metronidazole	65.96	>48	>1024.39	48	1.5	0.01	319.86	3	24	3
Amikacin	4.50	4	256.85	0.06	<0.01	1	8.12	0.19	0.75	0.25
Clindamycin	35.98	24	1024.39	10	1.5	0.07	131.93	3	127.93	1.5
Fluconazole	NT	NT	NT	NT	NT	NT	NT	NT	NT	257.85

NT: not tested; Microorganisms: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Proteus mirabilis, Vibrio cholerae, Streptococcus pyogenes, Salmonella paratyphi B, Candida albicans*

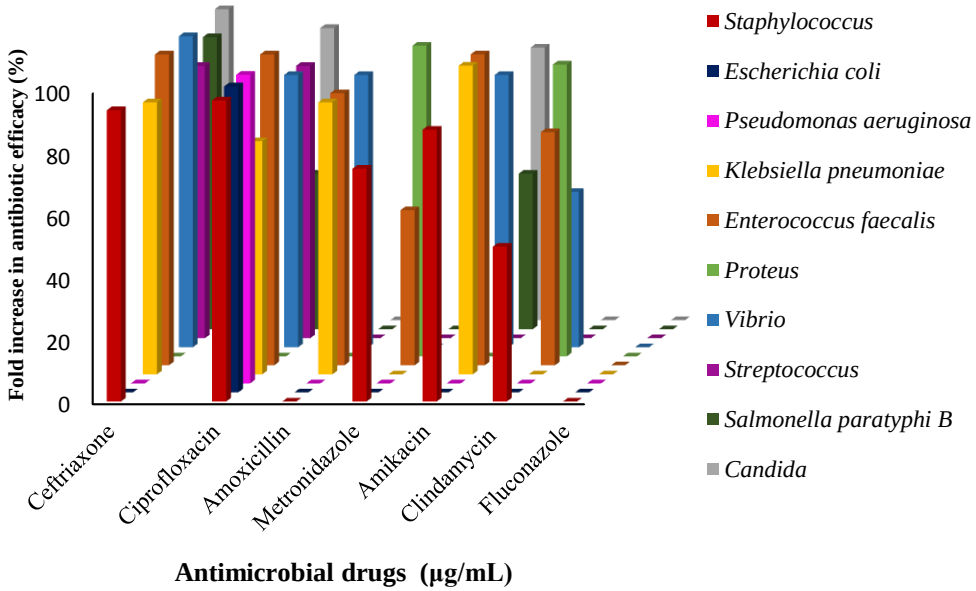

Figure 1. The percentage fold increase in the antimicrobial efficacy

The HT-SPOTi assay conducted in 96 microtiter plates screens a large number of diverse compounds against microorganisms to identify their antimicrobial potential and involves the incorporation of different concentrations of inhibitors into a nutrient agar medium followed by the spotting of a standardized number of cells onto the surface of the agar. Growth is assessed after incubation for a defined period (16-20 h) and the MIC value is recorded [10,11]. A combination of inhibitor compounds can be assayed simultaneously. Advantages of the HT-SPOTi method are its simplicity, rapidness, low cost, high efficiency and miniaturization

compared to traditional susceptibility methods [7]. According to existing literature, andrographolide has potent anti-bacterial activity and could be useful in preventing resistance. Studies conducted on andrographolide to investigate the potential mechanism of anti-infective action revealed that andrographolide could prevent the development of biofilms in *Staphylococcus aureus* in a concentration-dependent manner [12], decrease the production of virulence factors such as pyocyanin, proteolytic and elastase activity in *Pseudomonas aeruginosa* cultures [13], decreased bacterial adhesion, and the induced loss of bacterial integrity in *Escherichia coli* by disrupting the bacterial Quorum sensing system [14]. Also, by suppressing the action of bacterial efflux pumps, andrographolide restore the sensitivity of *Pseudomonas aeruginosa* cells to antibiotics [15].

In previous combination studies, Zeng X et al. investigated the synergistic effects of a novel derivative, 14-alpha-lipoyl andrographolide combined with conventional antibiotics; fosfomycin, streptomycin, azithromycin, erythromycin, gentamicin and ciprofloxacin against *Pseudomonas aeruginosa strains*. In their report, the activity of the antibiotics alone showed potent inhibition of cell growth than the 14-alpha-lipoyl andrographolide derivative. However, when the antibiotics were combined with the 14-alpha-lipoyl andrographolide derivative, their anti-infective and antibiofilm activities greatly increased [16]. Likewise, the lowest concentration of andrographolide that inhibited the growth of *Pseudomonas aeruginosa* in the present study was 109.38 µg/mL but combined with ciprofloxacin the minimum inhibitory concentration substantially decreased to 0.855 µg/mL.

Also, Moumita M. et al. reported that 14-deoxy-11,12-didehydroandrographolide at 0.1 mM (sub-MIC dose) demonstrated synergism when combined with azithromycin (6 µg/mL) or gentamicin (4 µg/mL) and inhibited 92% biofilm production against *Pseudomonas aeruginosa* [17]. In the present study, the fractional inhibitory concentration index (FICI) was adopted to evaluate the combinatorial effect of andrographolide with the tested antimicrobials, which can be synergistic, additive, indifferent or antagonistic [10,18]. Synergy is defined as a combined action of two or more agents that is larger than the expected additive effect of those agents. Antagonism is the absolute opposite of synergy; it occurs when a compound's combined effect is less than expected [19]. The antimicrobials tested had different mechanisms of action to represent the broad category of antimicrobial types. The results of this study indicated that andrographolide revealed proper combination effects with ceftriaxone, ciprofloxacin, and amikacin against some of the pathogens tested. The synergistic effect observed showed a greater effect than the combined effects of each constituent.

Combinations of antimicrobial agents provide many benefits such as enhancing antibacterial activity, minimizing dose-dependent side effects, reducing the time needed for long-term antimicrobial therapy and preventing the emergence of resistant microorganisms [5].

Although combination therapy could enhance antimicrobial efficacy, the use of andrographolide with antimicrobials should be done with caution as not all the antimicrobial drugs displayed synergic combination effects. Antagonistic interactions were observed when andrographolide was combined with amoxicillin, metronidazole, clindamycin and fluconazole against most of the tested microorganisms. Thus, a decreased activity was observed for the treatment combination with andrographolide compared to the effect of each individual constituent. Currently, the rapid increase in the consumption of herbal remedies worldwide was caused by several factors including the notion that all herbal products are safe [20]. However, from the results of this study, bioactive compounds from plants such as andrographolide could have antagonistic interactions with other antimicrobial drugs which can lead to serious adverse effects.

Conclusion

Andrographolide demonstrated selective inhibition of microorganisms when combined with antimicrobials and could be an effective strategy for reducing the emergence of drug-resistant strains. Further studies would be carried out in our laboratory to explore the mechanisms of the synergic interactions observed and evaluate the combined effects in living organisms.

Acknowledgments

The authors are grateful for the support and assistance of the Anti-infective Drug Discovery team, Department of pharmacology, KNUST.

Author contributions

Selase Ativui participated in the investigations, manuscript writing and editing; Cynthia Amaning Danquah was involved in the methodology, conceptualization and supervision, Simon Gibbons and Sanjib Bhakta participated in conceptualization and methodology; Michael Ofori and Peace Doe helped in data analysis. All authors have read and agreed to the published version of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

DMSO: dimethyl sulfoxide; FIC: fractional inhibitory concentrations; HT-SPOTi: high throughput spot culture growth inhibition; MIC: minimum inhibitory concentration