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Combination of *Momordica charantia L.* with oxytetracycline enhanced antibacterial and antibiofilm activities against some multidrug-resistant bacteria

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ABSTRACT

Background: *Momordica charantia L.*, the common name for bitter gourd, frequently used as a vegetable and in traditional medicine to treat wounds, peptic ulcers, parasites, and worms. Regarding of finding alternative ways to cure nosocomial infection caused by multidrug-resistant bacteria, bitter gourd in combination with some antibiotics may be a practical choice to reduce the cost of therapy and be devoid of side effects from antibiotics.

Objective: This study aimed to determine the antimicrobial, antibiofilm, and synergy effects of ethanol extract from bitter gourd in combination with conventional antibiotics, ampicillin, and oxytetracycline against some drug-resistant bacteria.

Materials and methods: The antimicrobial activity was tested by broth microdilution, and the lowest concentration that inhibits the visible growth of each microorganism was recorded as MIC. A checkerboard microdilution assay was designed to test the synergistic effect of bitter gourd extract. A crystal violet staining assay was carried out to test antibiofilm activity.

Results: The bitter gourd extracted by ethanol revealed antibacterial activity with a MIC range of 1.25-80 mg/mL. Synergistic effects of bitter gourd extract with ampicillin and oxytetracycline were effective against *P. mirabilis* and drug-resistant *P. aeruginosa* growth by FICI at 0.141 and 0.63, respectively. The results found that bitter gourd exhibited antibiofilm activities against *E. coli* ATCC 25922, drug-resistant *P. aeruginosa*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA) at 2-4 hours after starting inoculum and the inhibitory efficacy values were 37.62%, 71.14%, and 69.87%, respectively.

Conclusion: The ethanol extract from bitter gourd had antibacterial effect, synergy effect when mixed with ampicillin and oxytetracycline.

Introduction

Momordica charantia L., common name as bitter gourd or bitter melon, belongs to family Cucurbitaceae. It has been found in tropical areas of Asia, the Amazon, east Africa, and the Caribbean.[1] In developing countries like Brazil, China, Cuba, India, Mexico, and Thailand, all parts of bitter gourd, such as fruits, vines, leaves, and even roots, have been frequently used as a vegetable as well as a folk medicine for the treatment of wounds, peptic ulcers, diarrhea, diabetes, measles, and hepatitis.[2] By clinical studies, phytochemicals such as triterpene glycosides, furpyronecucurbitane A, goyaglycoside I, and charantagenin revealed anti-hepatic fibrosis activity and anti-hepatoma activity for the treatment of hepatic fibrosis or carcinoma.[3,4] Many biological active compounds found in bitter gourd included flavonoids, saponins, tannins,

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anthraquinones, and terpenoids revealed antimicrobial activity against *Eschericia coli, Staphylococcus aureus, Bacillus subtilis*.[5,6] In addition, a few preliminary have been reported anticancer, antimalarial, antihelmintic, antioxidant, anti-inflammatory, and immunomodulatory activities of bitter gourd, and especially bitter gourd extract could be an inhibitor against SARS-CoV-2.[1,2,7,8]

For centuries, the incidence of multidrug-resistant bacteria has risen and spread obviously because of antibiotic misuse and inappropriate consumption. Mainly, antibiotics are widely used to promote and prevent disease in agriculture and aquaculture. Commercial and conventional antibiotics such as tetracycline and ampicillin are unsuccessful in treating multi-drug-resistant infections.[9] Moreover, there is a lack of funding to search for novel antibiotics to treat nosocomial infection caused by *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* of which caused a wide range of chronic infections in patients with severe burns, organ transplants, cystic fibrosis, COVID-19, AIDS, or cancer.[10]

Some strategies, such as using natural compounds derived from medicinal plants and combining medicinal plants with antibiotics, are alternative methods to reduce the spread of nosocomial infection caused by multidrug-resistant bacteria. Many reports have shown the mechanism and synergistic effect of natural products such as caffeic acid with tetracycline and quercetin with amoxicillin against some bacteria.[11] Antibacterial and antibiofilm synergy effects of medicinal plants in combination with antibiotics may prevent adherence of planktonic bacterial cells to medical devices such as catheters and cardiac valves.[12] Nevertheless, only a few researchers declared that essential oils in combination with antibiotics inhibited the biofilm formation of multidrug-resistant bacteria.[12] Additionally, reducing antibiotics used for growth promotion in agriculture, livestock farming, and aquaculture may be a great deal of dawn-resistant incidence.

Thus, this investigation aimed to determine single-use and synergistic antibacterial and antibiofilm activities of ethanol extract from bitter gourd and bitter gourd in combination with some conventional antibiotics against some multidrug-resistant and opportunistic bacteria. Likewise, increasing the effectiveness of traditional antibiotics with bitter gourd will be a model to elucidate the pharmacological action of bitter gourd for curing infectious diseases against multidrug-resistant bacteria.

Materials and methods

Bacteria strains

Bacillus cereus, *B. subtilis*, *Klebsiella pneumoniae* and *Proteus mirabilis* were isolated from clinical specimens. The standard strain of the experiment was *Escherichia coli* ATCC 25922. *A. baumannii*, resisted to aminoglycosides, β-lactam and quinolone, *P. aeruginosa* Code1-375/04-2013, resisted to aminoglycosides, β-lactams, carbapenem, quinolone and Methicillin Resistant *Staphylococcus aureus* (MRSA) were confirmed and characterized by API 20 NE System. Also, *A. baumannii* and *P. aeruginosa* were confirmed *MexA* and *MexB* genes by Polymerase Chain Reaction.[13]

Momordica charantia L. extract

The young leaves of bitter gourd (characterized as voucher number Buu-Cho/64-3) were collected from February to March 2022 in Chonburi province, Thailand. The fresh young leaves were dried under shade and sliced into tiny pieces. The 500 mg of ground leaves were extracted with 80% alcohol for 4 days.[14] A rotary evaporator lyophilized the suspension of leaf extract. The crude bitter gourd extract with a 3.01% yield was kept in a protected light bottle at 4 C before being tested.

Minimum inhibition concentration (MIC) determination

The broth microdilution susceptibility test and lowest concentration inhibiting each microorganism's visible growth were recorded as MIC. as described by the Clinical and Laboratory Standard Institute (CLSI).[15] Briefly, the 100 µL of 80 mg/mL bitter gourd extract was diluted for serial two-fold dilution (ranging from 0.625 mg/mL to 80 mg/mL) by 100 µL of Mueller Hinton Broth (MHB). Then, 100 µL of $1.5x10^5$ CFU/mL bacterial suspension was added to 100 L of MHB in a microtiter plate. The 96-well microtiter plate was incubated at 37°C for 18-24 h, and OD600 was measured by a microplate reader (VersaMax, U.S.A.). The 100% ddH_2O was negative control, and two antibiotics, ampicillin, and tetracycline, were positive control, and all treatments were determined in triplicate. The lowest concentration that prevented 100% viable growth of OD600 was recorded as the MIC. All MICs were determined in triplicate.

Synergistic effect determination

The checkerboard broth microdilution assay was modified to test the synergistic effect of bitter gourd extract on bacterial growth.[16] The 50 µL of two-fold serial concentrations of bitter gourd extract and 50 µL of two-fold serial concentrations of ampicillin and oxytetracycline ranging from 1.25 mg/mL to 80 mg/mL were tested by broth microdilution susceptibility test. The triplicate sampling of synergistic MIC was analyzed. The checkboard microdilution results of synergistic effect were compared to MICs of a single dose of bitter gourd extract and antibiotics. The fractional inhibitory of synergistic effects was calculated. FICIs were interpreted by the following: a synergism as FICI index of FICI≤0.5, a partial synergism, as FIC index of 0.5<FICI<1, an additive effect, as FICI index of FICI=1, an indifferent effect, as FICI index of 1<FICI≤4, an antagonism, FICI index of FICI>4.[16]

Antibiofilm activity assay

The antibiofilm method was modified from Zheng *et al.* (2021).[17] Briefly, 100 µL aliquot of sample / positive control *(*oxytetracycline) was mixed with 100 µL of $1x10^6$ CFU/mL of bacterial culture in flat-bottomed 96-well microtiter plates, and 100 µL of Tryptic Soy broth (TSB, Difco, U.S.A.) were applied to microplates. Next, microplates were incubated for 2-24 h. At an interval, each

well of adherent biofilm of bacteria was stained with 1% crystal violet for 15 minutes and fixed with 200 µL of 100% methanol for 15 minutes. The stain of bacterial biofilm was solubilized by 150 µL of 33% glacial acetic acid. Finally, the soluble biofilm of bacteria was measured in the OD600 by a microplate reader VersaMax (U.S.A.). All treatments were done in triplicate. The absorbance and percentage inhibition of biofilm of each strain were determined by triplicate sampling as equation 1.

$$\% \text{ inhibition} = (OD_{control} - OD_{treatment})/OD_{control} 100\times \quad (1)$$

$OD_{control}$: optical density of bacteria without compound or antibiotic.

$OD_{treatment}$: the optical density of bacteria mixed with compound.

Results

Determination MICs of bitter gourd extract

The MIC values of single-use bitter gourd extract or single use or ampicillin/oxytetracycline were summarized in Table 1. The bitter gourd extracts inhibited the growth of all bacterial strains with 80 mg/mL of MIC except for *B. cereus*. The bitter gourd extract showed the best activity against MRSA. In addition, ampicillin and tetracycline activity showed significantly better than bitter gourd extract.

Table 1. The antibacterial activity of bitter gourd extract and antibiotics against some opportunistic bacteria.

Bacteria	Minimum Inhibitory Concentration (MIC) (mg/mL)		
	Momordica charantia	Oxytetracycline	Ampicillin
A. baumannii	80	40	80
B. cereus	>80	20	40
B. subtilis	20	2.5	>80
E. coli ATCC 25922	80	80	80
K. pneumoniae	80	1.25	1.25
MRSA	1.25	20	1.25
MDR *P. aeruginosa*	80	2.5	10
P. mirabilis	80	20	1.25

Synergistic effect of bitter gourd extract in combination with antibiotics

The synergistic effect of the bitter gourd extract in combination with both ampicillin and oxytetracycline (Table 2) was observed against all bacteria except *A. baumannii* and *E. coli* ATCC25922. The best synergism effect of bitter gourd in combination with tetracycline was shown against *P. mirabilis* (FICI = 0.141) and multidrug-resistant *P. aeruginosa* (FICI = 0.63). Unfortunately, synergistic effect of ampicillin or oxytetracycline mixing with bitter gourd extract against *A. baumannii, B. cereus*, *B. subtilis* and *E. coli* ATCC25922 was impractical (data was not shown).

Table 2. The synergistic effect of bitter gourd extract in combination with antibiotics against bacteria.

Bacteria	The synergistic effect of bitter gourd extract in combination with antibiotics			
	bitter gourd extract + Ampicillin		bitter gourd extract + Oxytetracycline	
	FICI	Interpretations	FICI	Interpretations
K. pneumoniae	2.016	Indifferent	2.016	Indifferent
P. mirabilis	-	-	0.141	Synergism
MDR *P. aeruginosa*	0.63	Partially synergism	-	-
MRSA	2	Indifferent	1.063	Indifferent

Note: - represented as no FICI. a synergism, as FIC index of ≤0.5, a partial synergism, as FIC index of 0.5< FICI<1, an additive effect, as FICI index of FICI=1, an indifferent effect, as FICI index of 1<FICI≤4, an antagonism, FICI index of FICI>4

Antibiofilm activity of bitter gourd extract

The inhibitive effect of biofilm formation of *E. coli* ATCC 25922, MRSA, and drug-resistant *P. aeruginosa* was shown in Figure 1-3, and at the concentration of 80 mg/mL, a single dose of bitter gourd extract indicated significant antibiofilm activity against all tested strains at 2-4 h and % inhibition ranging from 37.14%-71.14% ($p<0.05$). A combination of 80 mg/mL bitter gourd extract and 80 mg/mL oxytetracycline showed significantly better antibiofilm activity than a single dose of bitter gourd extract or oxytetracycline at 2-8 h against MRSA and multidrug-resistant *P. aeruginosa*. However, bitter gourd extracts alone or combined with ampicillin showed no effect (data not shown).

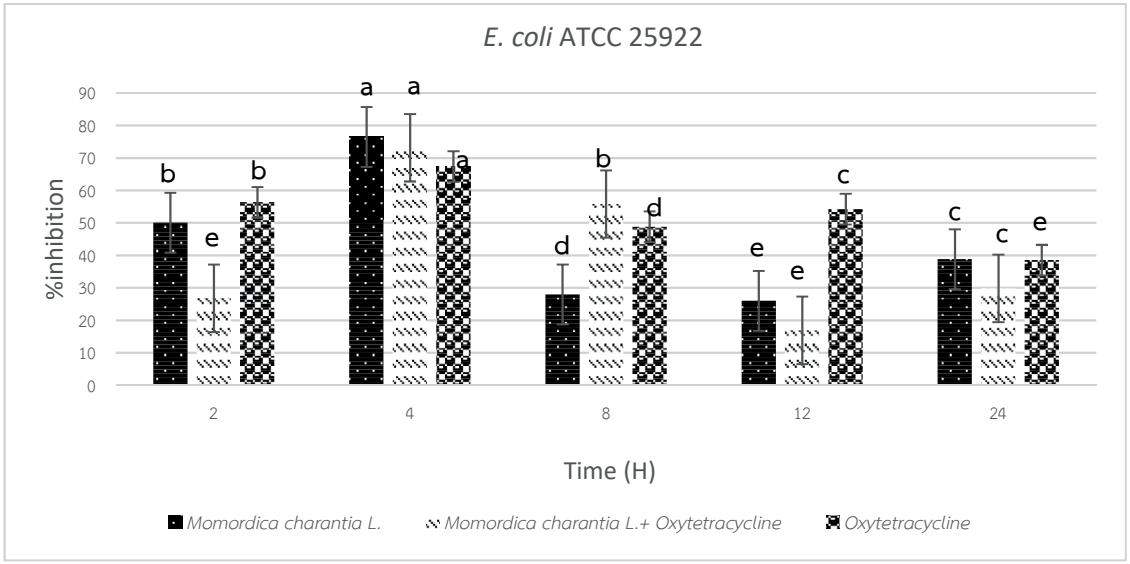

Figure 1. *Effect of 80 mg/mL bitter gourd extract in combination with 80 mg/mL oxytetracycline on biofilm formation of E. coli ATCC 25922. *a-e significant different (p≤0.05).*

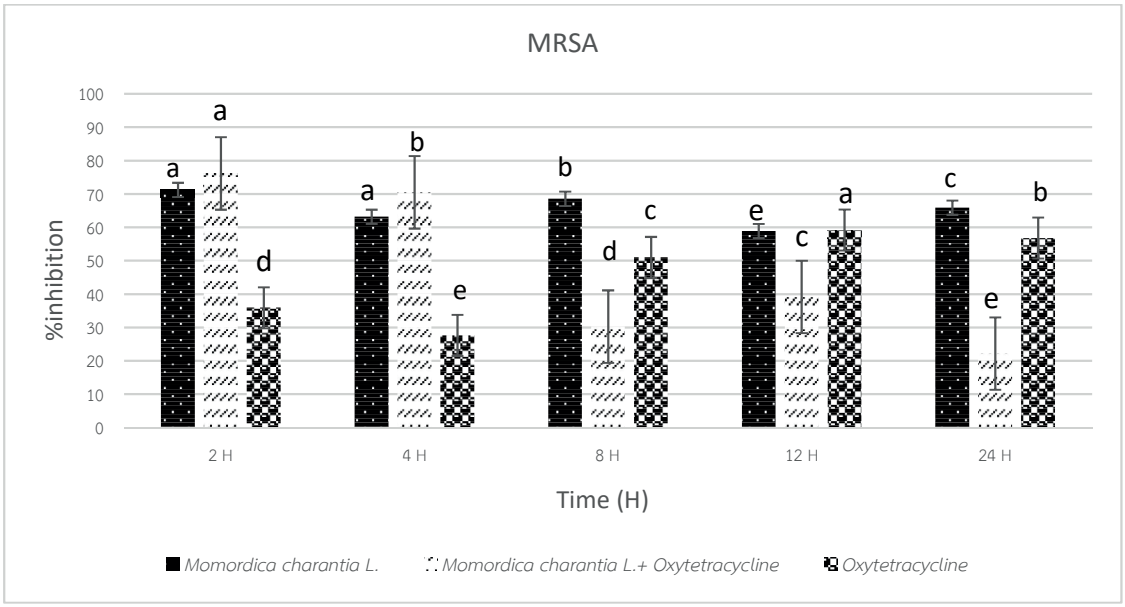

Figure 2. *Effect of 80 mg/mL bitter gourd extract in combination with 80 mg/mL oxytetracycline on biofilm formation of MRSA. *a-e significant different (p≤0.05).*

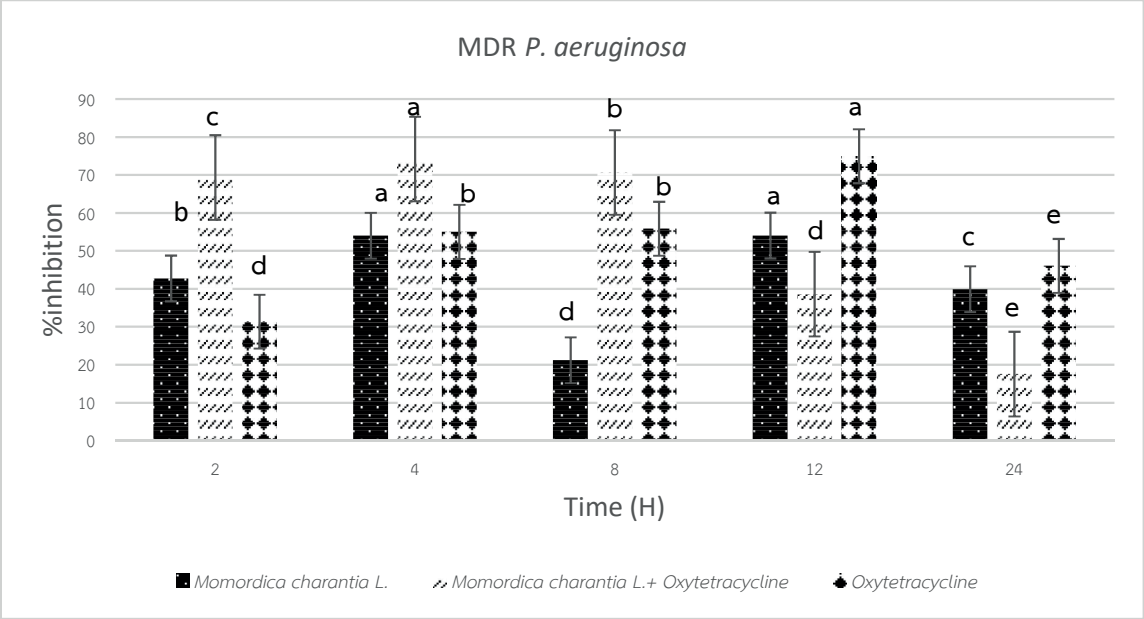

Figure 3. *Effect of 80 mg/mL bitter gourd extract in combination with 80 mg/mL oxytetracycline on biofilm formation of multidrug resistant P. Aeruginosa.* $^{*a\text{-}e}$*different superscript letter within Each bar are represented significantly different (p≤0.05).*

Discussion

Multidrug-resistance causes severe problems and impacts the treatment of infectious diseases. Accumulation of opportunistic bacteria, including *A. baumannii*, *E. coli*, *K. pneumoniae*, MRSA, and *P. aeruginosa*, caused by innate genetics and physiological transmission.[9] It was the most troublesome problem in pharmacological industries and clinical treatment of infected patients that standard antibiotics, ampicillin, and oxytetracycline right now were inactive and could not be helpful to treat infected patients.[10] The bitter gourd may solve the problem of how to bring conventional antibiotics back to treat bacterial coinfection with COVID-19 and to reduce the side effect of these antibiotics.[7] In this research, bitter gourd extracted showed a well-known antibacterial activity against some multidrug-resistant bacteria due to many secondary metabolites, such as tannins, flavonoids, and alkaloids, which had many biological actions.[18] In some reports, the antibacterial result declared that ethanol extract of bitter gourd showed only antibacterial activity against *S. aureus* when using MIC as 1.25 µg/mL as the same result, this study showed antibacterial effect against MRSA (MICs of 1.25 mg/mL).[19]

The synergistic effect of this report indicated that bitter gourd extract in combination with both ampicillin and oxytetracycline exhibited the highest efficiency against *P. mirabilis* and drug-resistant *P. aeruginosa*. As the result of Wagner and Ulrich-Merzenich, the synergistic effect of bitter gourd extract may improve solubility, reabsorption rate of ampicillin and tetracycline and inhibit proteins such as enzyme and transport protein, ribosome, DNA, and RNA synthesis of gram-negative bacteria.[20] Bitter gourd extract contains phenolic compounds like the essential oil of *Thymus vulgarism*, such as p-cymene, γ-terpinene, and thymol so that bitter gourd extract could inhibit the growth of *E. coli*, *K. pneumonia*, and *S. aureus*.[21] In particular, Mallotojaponin B, in combination with chloramphenicol, showed the same synergistic effect with FICI 0.393 against MRSA and indicated the mechanism that caused alteration and destruction of the cell membrane of MRSA.[22] In addition, this was consistent with the finding of Navy *et al.* that oxytetracycline in combination with epigallocatechin gallate (EGCG) could inhibit the growth of *S. aureus*.[23] Likely, Vipin *et al*. found that quercetin with levofloxacin, ceftriaxone, gentamycin, tobramycin, and amikacin showed 68 to 85% reducing the growth of multidrug-resistant *P. aeruginosa*.

The initial stage of biofilm formation was a target goal to inhibit bacterial growth and adhere to their target surface. In this study, results indicated that bitter gourd extract in combination with oxytetracycline showed potential antibiofilm activity against all bacterial strains. Based on our findings on the antibiofilm activity of drug-resistant bacteria, the highest dose of bitter gourd extract and oxytetracycline showed a synergistic effect with MIC (80 mg/mL). Based on the study, the lowest MIC values of 25 µg/mL and 250 µg/mL of the inhibitory effect of grape seed fruit were used. This study showed better activity than the antibiofilm activity of bitter gourd against *S. aureus* and *E. coli*.[24] Moreover, Vipin *et al*. studied the synergistic activity of quercetin at 125 µg/mL and showed a synergistic effect with ½ × MIC or ¼ × MIC of levofloxacin (125 µg/mL) against *P. aeruginosa*.[25] Thus, bitter gourd extracts enhanced antibiofilm activity of oxytetracycline against *E. coli*, MRSA, and *P. aeruginosa* may be closely related to actions of flavonoids, saponins, tannins, anthraquinones as the primary component found in bitter gourd leave.[6,26]

This study showed a successful report that bitter gourd extract could synergize the antibacterial activity

of ampicillin and oxytetracycline to inhibit opportunistic and drug-resistant bacteria growth. Also, the success of bitter gourd extract in combination with oxytetracycline may be helpful to prevent some drug-resistant bacterial growth and biofilm formation. Thus, bitter gourd may be a choice for the therapeutic management of nosocomial infection. However, no mechanism has been explained for the antimicrobial activity of bitter gourd extract. Toxicity and interaction of bitter gourd with oxytetracycline may be tested in the future.

Conclusion

In this study, young leaves of bitter gourd extract can synergize oxytetracycline's antibacterial and antibiofilm activities to inhibit the growth of some opportunistic and drug-resistant bacteria, such as *E. coil*, MRSA, *and P. aeruginosa*. A combination of antibiotics and bitter gourd may be a choice for therapeutic management of caring for infectious diseases and reducing the overuse of antibiotics in environments.

Conflict of interest

The authors declare no conflict of interest.

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