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Original Article

Antibacterial Synergistic effect and antibiofilm activity of 3-epi-lupeol from *Glochidion eriocarpum* against opportunistic Bacteria

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Abstract

The 3-epi-lupeol, a natural compound extracted from *Glochidion eriocarpum* (Muell. Arg. Kurz), has been widely demonstrated to exhibit antibacterial, antioxidant, and anticancer activities. In this study, the antibacterial and antibiofilm activities of 3-epi-lupeol, in combination with ampicillin and oxytetracycline, against several opportunistic bacteria were investigated. The results indicated that a single dose of 3-epi-lupeol exhibited potent antibacterial activity against *Bacillus cereus, B. subtilis, Klebsiella pneumoniae, Proteus mirabilis, P. vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus* with MICs ranging within 128-1024 μM. The combination of 3-epi-lupeol and oxytetracycline exhibited a synergistic antibacterial effect against *P. aeruginosa* (FICI = 0.27) and a partially synergistic effect against *E. coli* ATCC 25922 (FICI = 0.75). Confirmed by time kill assay, the synergism effect of 1⁄32 MIC -1⁄64 MIC 3-epi-lupeol mixed with 1⁄4 MIC - ½ MIC oxytetracycline significantly reduced by 1.5 $\log_{10}$ the bacterial counts of *E. coli* ATCC 25922 and *P. aeruginosa* at 2-8 hours after starting inoculum ($p \leq 0.05$). Finally, the 3-epi-lupeol showed significant antibiofilm activity against *A. baumannii, E. coli* ATCC *25922* and *P. aeruginosa* ATCC *27853* at 2-12 hours at interval timing ($p \leq 0.05$).

Keywords: antibacterial activity, synergistic effect, antibiofilm activity, 3-*epi*-lupeol, *Glochidion eriocarpum* Champ. ex Benth.

1. Introduction

Antibiotic resistance gained by bacteria is a serious problem with significant clinical impact on the treatment of infectious diseases. So far, the incidence of multi-drug-resistant opportunistic pathogens has clearly increased (Schroeder, Brooks, & Brooks, 2017; Walsh, Gales, Laxminarayan, & Dodd, 2023). Additionally, bacterial secondary infections and co-infections in patients with COVID-19 are predicted to increase the severity of the disease and, consequently, contribute to higher mortality rates in COVID-19 infections (Farrell *et al.*, 2021). Thus, conventional antibiotics have not been successful in treating multi-drug-resistant opportunistic pathogens (Chessman *et al.*, 2017; Subramani *et al.*, 2017). Addressing antibiotic resistance is a critical challenge, as new classes of antibiotics are developed slowly and are costly to produce. Therefore, it is essential to explore natural products as alternatives to ineffective antibiotics and to investigate new therapeutic strategies, such as combining active compounds with conventional antibiotics to treat opportunistic pathogens. Recent studies have indicated that terpenes and their derivatives, such as lupeol, exhibit significant antibacterial activity against multi-drug-resistant bacteria (Mahizan *et al.*, 2019).

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Glochidion eriocarpum Champ. ex Benth., a monoecious shrub, is commonly found in the forests of China, Thailand, Taiwan, and Vietnam. In folk medicine, various parts of *G. eriocarpum*, particularly the roots and leaves, are used to treat conditions such as urticaria, mastitis, menorrhagia, dysentery, enteritis, and toothache (Zhang *et al*., 2020). According to a previous report, 3-epi-lupeol, isolated from the leaves, roots, and stems of *G. eriocarpum*, demonstrated antioxidant activity and induced apoptosis in cancer cells (Puapairoj *et al*., 2005). Lupeol, a compound found in many fruits and medicinal plants, has also been shown to exhibit anticancer activity in both *in vitro* and *in vivo* assays against various cancer cell lines by inducing apoptosis through the Ras signaling pathway. For instance, it has been effective against human pancreatic adenocarcinoma cells (Liu *et al*., 2016; Siddique & Saleem, 2011). Additionally, lupeol has been reported to possess anti-arthritis, antimicrobial, anti-diabetic, and anti-inflammatory activities in animal models (Siddique & Saleem, 2011). Furthermore, lupeol has potential therapeutic and preventive applications, such as cardioprotective, hepatoprotective, nephroprotective, and skin-protective properties (Siddique & Saleem, 2011; Zhang *et al*., 2020). A recent study also indicated that lupeol derivatives isolated from birch bark could promote wound healing by preventing tissue degradation and inhibiting protein oxidation caused by free radicals associated with skin aging (Malinowska *et al*., 2021). In preclinical studies, lupeol demonstrated antibacterial activity against several opportunistic bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *S. paratyphi*, *Salmonella typhi*, and *Klebsiella pneumoniae* (Rosandy *et al*., 2021; Zhang *et al*., 2020).

The first line of defense against antimicrobial resistance is to eradicate bacteria attached to the surface of human tissues and inhibit biofilm formation by resistant bacteria (Zhou *et al*., 2015). Several effective antimicrobial agents, such as cisplatin, docosahexaenoic acid, phenazines, and quinolines, have been shown to reduce bacterial biofilm formation (Verderosa, Totsika, & Fairfull-Smith, 2019). A previous report indicated that compounds like lupeol, betulinic acid, and ursolic acid inhibited biofilm formation by *Enterococcus faecalis*, *Staphylococcus aureus*, and *S. epidermidis* (Silva *et al*., 2019). Additionally, the combination of bicarinalin with colistin sulfate was found to eradicate bacteria attached to surfaces and prevent matrix formation by *Acinetobacter baumannii* (Eales *et al*., 2018).

Due to the ineffectiveness of conventional antibiotics such as ampicillin and oxytetracycline, there are limited options for treating drug-resistant bacteria (Schroeder *et al*., 2017). Thus, an alternative approach to enhance the efficacy of conventional antibiotics, like ampicillin and oxytetracycline, is to combine them with natural products. The objective of this study is to evaluate the antibacterial activity, antibiofilm activity, and synergistic effects of 3-epi-lupeol, a compound characterized from *G. eriocarpum*, against opportunistic bacteria. The results may lead to the discovery of a new natural compound that could enhance the effectiveness of conventional antibiotics, such as ampicillin and oxytetracycline, while also exhibiting antibiofilm activity to help eradicate the initial stages of chronic infections caused by opportunistic and drug-resistant bacteria.

2. Materials and Methods

2.1 Plant material derived from *G. eriocarpum*

The 3-epi-lupeol, a derivative of lupanes from the roots of *Glochidion eriocarpum* (voucher specimen No. BKF147874), was characterized as previously described by Puapairoj *et al*. (2005). The 2 kg sample of *G. eriocarpum* roots and stems was extracted using 100% hexane and separated by incremental gradient elution via column chromatography. Crystals were formed, and the pure compound was identified as a lupane derivative through 1H NMR, 13C NMR, DEPT-90, and DEPT-135 spectra, with a molecular weight of 426. The structure of 3-epi-lupeol is shown in Figure 1.

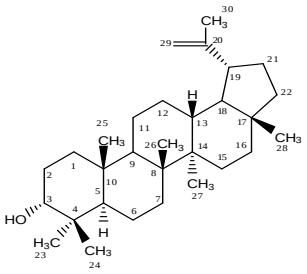

Figure 1. Structure of 3-*epi*-lupeol (M.W. 426) derived from roots of *G. eriocarpum*

2.2 Bacterial strains and culture

A standard strain, *E. coli* ATCC 25922 and seven opportunistic bacteria; *Bacillus cereus*, *B. subtilis*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Staphylococcus aureus*, were obtained from Department of Microbiology, Burapha University, Chonburi, Thailand. The three drug resistant strains were *A. baumannii*, which lacked sensitivity to aminoglycosides, β-lactam and quinolone; and *P. aeruginosa* Code 1-375/04-2013, was resistant to aminoglycosides, β-lactams, carbapenem, and quinolone; and the Methicillin Resistant *Staphylococcus aureus* (MRSA). These were provided by the Chonburi Hospital, Chonburi Province, Thailand. All the bacterial strains were confirmed and characterized by API 20 NE System. Also, the drug resistant *MexA* gene, forward primer 5' CAG CAG CTC TAC CAG ATC GAC 3' and reverse primer 5'TTC GGT AAC CAG CCA CTT GT3' and *MexB* gene, forward primer 5'AGG TCC AGG TGC AGA ACA AG 3' and reverse primer 5' GGA ATC GAC CAG CTT TCG TA 3' were used to identify drug resistant genes from *A. baumannii* and *P. aeruginosa* by polymerase chain reaction (Nehme, Li, Elliot, & Poole, 2004). The PCR products showed 1,175 base pairs and 2,300 base pairs for *MexA* and *MexB* genes, respectively.

2.3 Evaluation of minimum inhibitory concentration (MIC)

The broth microdilution assay was modified from the Clinical and Laboratory Standards Institute (CLSI, 2016). Bacterial cultures were grown in Mueller-Hinton Broth. The

100 μL of 1 x10^8 colony forming unit per mililiter (CFU/mL) bacterial suspension, estimated by an 0.5 MacFarland standard, was mixed with 100 μL 3-epi-lupeol and standard antibiotics in microtiter plate (Coning, U.S.A.) The 3-epi-lupeol and antibiotics were diluted by serial two-fold dilutions to cover a range from 2 μM to 2048 μM by 100 μL of MHB. After mixing well, all treatment of samples were incubated at 37ºC for 24 hours. Next, the absorbance at 600 nm (OD600) of each well with treated cultures was measured using a microplate reader (VersaMax, U.S.A.) to assess bacterial growth. The lowest concentration at which no visible growth was observed was recorded as the MIC. The antibacterial activity of 3-epi-lupeol was compared to that of the antibiotics, ampicillin and oxytetracycline (positive controls), and 100% DMSO and ddH_2O (negative controls). Each treatment of 3-epi-lupeol and antibiotics was performed in triplicate.

2.4 Synergistic effect of 3-epi-lupeol and antibiotics

The broth microdilution assay was used to assess the synergistic effect. 3-epi-lupeol (ranging from 2 μM to 2048 μM) and antibiotics (2 μM to 2048 μM) were mixed and tested in a checkerboard assay format (Hall *et al*., 1983). A 50 μL aliquot of 3-epi-lupeol was mixed with 50 μL of the antibiotics and added to a microplate, resulting in a final volume of 100 μL per well. Then, 100 μL of a bacterial suspension (1 × 10^8 CFU/mL) was added to each well. The microplate was incubated at 37°C for 24 hours, and all treatments were performed in triplicate. Bacterial growth was measured using a microplate reader at OD_{600}. The concentration of synergistic MIC was selected along with the wells that had no visible growth to evaluate the MIC of 3-epi-lupeol in combination with antibiotics. For two antibacterial agents, individual MIC_A, individual MIC_B, the MIC $_{(A \text{ in a present of B})}$, the MIC $_{(B \text{ in a present of A})}$ were recorded and the fractional inhibitory concentration (FIC) was quantified. FICA and FICB were calculated using equations 1–3. The sum of FICA and FICB was then used to determine the synergistic effect, which was interpreted using the Fractional Inhibitory Concentration Index (FICI), as described in equation 3. FICI values were interpreted as follows: synergism for FICI ≤ 0.5, partial synergism for 0.5 < FICI < 1, an additive effect for FICI = 1, an indifferent effect for 1 < FICI ≤ 4, and antagonism for FICI > 4 (Hall, Middleton, & Westmacott, 1983).

$FIC_A = MIC_{(A \text{ in a present of B})} / MIC_A$ (1)

$FIC_B = MIC_{(B \text{ in a present of A})} / MIC_B$ (2)

$FICIs = FIC_A + FIC_B$ (3)

2.5 Time kill assay

The time-kill assay was conducted to monitor bacterial growth over 48 hours. A 500 μL inoculum of 1.5 × 10^8 CFU/mL was mixed with 500 μL of the compound combination or antibiotics, then added to 10 mL of Mueller-Hinton Broth (MHB). The bacterial cultures were incubated at 37°C for 2 to 48 hours. At various time intervals (2, 4, 8, 16, 24, and 48 hours), viable bacterial counts were determined by plating 100 μL of ten-fold serially diluted bacterial solutions onto Nutrient Agar (NA) (Difco, U.S.A.) plates. All treatments were performed in triplicate. The plates were incubated at 37°C for 24 hours, and colony counts were used to determine bacterial survival. The number of surviving bacteria was recorded as log colony-forming units per milliliter (log CFU/mL), and the effectiveness of antimicrobial activity was calculated using equation 4.

The effectiveness of antimicrobial activity (EAA) was calculated by equation 4:

$$EAA\ (\%) = \frac{N_0 - N_E}{N_E} \times 100 \quad (4)$$

N_0 and N_E were the number of control bacteria without compound or antibiotic and with compound or antibiotic, respectively.

2.6 Antibiofilm assay

Antibiofilm activity was assessed using the crystal violet staining method, as described by (Zhang, Liu, Lei, Zhou, & Long, 2020). Briefly, 100 μL of a 1MIC of the compound or ½MIC of the positive control (oxytetracycline) was added to flat-bottomed 96-well microplates. Then, 100 μL of a 1 × 10^6 CFU/mL bacterial culture and 100 μL of Tryptic Soy Broth (TSB, Difco, U.S.A.) were added to each well. The microplates were incubated without shaking for 4, 8, 12, 24, and 48 hours. At each point, bacterial biofilms were stained with 1% crystal violet and then washed with phosphate-buffered saline (PBS) to remove unstained bacteria. The microplates were air-dried at room temperature for 10 minutes. Next, the stained biofilms were fixed by adding 200 μL of 100% methanol and incubating for 15 minutes. Finally, the biofilm was solubilized with 150 μL of 33% glacial acetic acid. The solubilized biofilm was measured using a microplate reader (VersaMax, U.S.A.) at OD_{600}. The absorbance and percentage inhibition of biofilm formation were calculated using equation 5.

$$\% \text{ inhibition} = (OD_{control} - OD_{treatment}) / OD_{control} \times 100 \quad (5)$$

$OD_{control}$ was the optical density of bacteria without compound or antibiotic and $OD_{treatment}$ was the optical density of bacteria mixed with compound.

2.7 Statistical analysis

Bacterial growth and antibiofilm activity were analyzed using a two-way analysis of variance (ANOVA), with treatment and time as the variables. Differences among the means were assessed using Duncan's multiple range test, and statistical significance required that $p \leq 0.05$. All analyses were performed using Minitab software version 18 (Minitab Pty Ltd, Sydney, Australia).

3. Results and Discussion

3.1 Antibacterial activity by microdilution assay

The 3-epi-lupeol extracted from *G. eriocarpum* demonstrated effective antibacterial activity against eight strains of opportunistic bacteria but had no effect against drug-resistant bacteria, such as drug-resistant *A. baumannii*, MRSA, and *P. aeruginosa* (Table 1). *B. subtilis*, *P. mirabilis*,

Table 1. Antibacterial activities of 3-*epi*-Lupeol from *G. eriocarpum*, and the two antibiotics Ampicillin and Oxytetracycline

Bacteria	Strains	MIC (μM)		
		3-*epi*-Lupeol	Ampicillin	Oxytetracycline
Gram positive	*B. cereus*	512	128	128
	B. subtilis	128	128	128
	MRSA	>2048	>2048	512
	S. aureus	128	128	128
Gram negative	Drug-resistant *A. baumannii*	>2048	>2048	>2048
	E. coli ATCC 25922	2048	64	16
	K. pneumoniae	512	128	1024
	P. mirabilis	128	512	128
	P. vulgaris	512	1024	512
	P. aeruginosa	1024	>2048	64
	Drug-resistant *P. aeruginosa*	>2048	>2048	>2048

and *S. aureus* were the most susceptible strains to 3-epi-lupeol. The antibacterial activities of 3-epi-lupeol, Ampicillin, and Oxytetracycline against all opportunistic bacteria showed significant differences ($P \leq 0.05$). Compared to the antibacterial activities of conventional antibiotics, the antibacterial activity of 3-epi-lupeol was similar to that of Ampicillin and Oxytetracycline, with MICs ranging from 128 to 512 μM (54–218 μg/mL).

Reducing the emergence of drug-resistant bacteria is crucial for successfully treating infectious diseases. Historically, antibiotic therapy has been challenging due to the limited number of antibiotic classes available. Therefore, a promising strategy to combat drug-resistant bacteria is to use antibiotics in synergy with natural compounds. This approach is the focus of our research, aiming to identify new compounds that enhance the efficacy of conventional antibiotics against drug-resistant bacteria.

In this study, the single use of 3-epi-lupeol exhibited antibacterial activity similar to that of Ampicillin and Oxytetracycline against several bacteria, but not for the drug-resistant strains. Our results agree with those of a study on lupeol extracted from *Acokanthera oppositifolia*, which showed significant antibacterial activity against *P. aeruginosa* and *E. coli* (MICs of 7.81 and 0.24 μg/mL, respectively). However, 3-epi-lupeol demonstrated lower activity against *P. aeruginosa* (MIC = 436.2 μg/mL) and *E. coli* ATCC 25922 (MIC = 872.45 μg/mL) compared to lupeol extracted from *A. oppositifolia* (El Sayed *et al*., 2016). Gallo and Sarachine (2009) reported antibacterial activity of lupeol against *E. coli* ATCC 25922 (MIC = 250 μg/mL) and *P. aeruginosa* ATCC 27853 (MIC = 250 μg/mL), while showing no antibacterial activity against *Shigella* spp. and *S. aureus* (MIC > 200 μg/mL) (Gallo & Sarachine, 2009). However, in our results, the MIC of 3-epi-lupeol against *E. coli* (872.45 μg/mL) showed less antibacterial activity compared to triterpenes such as lupeol and betulinic acid (Mahizan *et al*., 2019). Mahizan *et al*. (2019) proposed the hypothesis that carvacrol and thymol (some monoterpene derivatives) may cause disintegration of the outer membrane and destruction of the cell membrane in gram-negative bacteria (Mahizan *et al*., 2019). 3-epi-lupeol, as one of the triterpene derivatives, may play a role in bacterial efflux pumps and the disruption of bacterial cell membranes (Mahizan *et al*., 2019). One proposed mechanism of terpenes is that they interfere with ion transport systems and disrupt phospholipids. Another proposed mechanism suggests that terpenes interfere with ion transport systems and disrupt the phospholipid bilayer of the bacterial cell membrane (Gupta & Birdi, 2017).

3.2 Synergistic effects of 3-epi-lupeol

Many classes of antibiotics such as ampicillin and the first generation oxytetracycline currently suffer from resistant strains and have critical inconveniences in treatment of bacterial infections. Synergism of Ampicillin and Oxytetracycline mixed with a natural product such as 3-epi-lupeol is critical in the challenge to control drug resistant bacteria. Thus, we investigated synergistic effects of 3-epi-lupeol in combination with Ampicillin and Oxytetracycline, and the results are presented in Tables 2 and 3. Oxytetracycline mixed with 3-epi-lupeol revealed the best synergistic effect against *P. aeruginosa* (FICIs of 27.0), and showed partially synergistic effect against *E. coli* ATCC 25922 (FICIs of 0.75) whereas Ampicillin mixed with 3-epi-lupeol had no synergistic effect against drug resistant and opportunistic bacteria (data not shown). Likewise, 3-epi-lupeol has the potential to increase Oxytetracycline activity when using ½ MIC to ¼ MIC Oxytetracycline in combination with ¼ MIC 3-epi-lupeol, as this enhanced antibacterial activity against *E. coli* ATCC 25922 and *P. aeruginosa*, respectively (Table 3).

The synergistic result is that 3-epi-lupeol potentially enhanced Oxytetracycline activity against *E. coli* ATCC 25922 and *P. aeruginosa*. The 3-epi-lupeol in combination with Oxytetracycline may cause disruption of bacterial cell membrane, alter the cellular respiration of bacteria and inhibit the expression of some virulence factors such as enzymes and toxins of gram-negative bacteria (Mahizan *et al*., 2019). As a similar outcome, the synergism of diterpene extract from leaves of *Polyathia longifolia* and 16α-Hydroxycleroda-3,13 (14)Z-dien-15,16-olide increased antibacterial efficacy of Oxytetracycline and oxacillin against MRSA (Mahizan *et al*., 2019). Our result agrees with Wang *et al*. (2016) reporting that oleanolic acid and ursolic acid showed synergistic effect with Gentamicin and Kanamycin against *A. baumannii* (Zacchino, Butassi, Cordisco, & Svetaz, 2017). Our data suggest that triterpenoids may potentiate antibiotics, particularly by enhancing the antibacterial activity of Oxytetracycline.

Table 2. Synergism of 3-*epi*-Lupeol combined with Oxytetracycline against bacteria *A. baumannii*, *E. coli* ATCC 25922, *P. aeruginosa* and drug-resistant *P. aeruginosa*.

Bacteria	The synergistic effect of 3-*epi*-Lupeol combined with antibiotics			
	3-*epi*-Lupeol combined with Ampicillin		3-*epi*-Lupeol combined with Oxytetracycline	
	FICIs	Interpretation	FICIs	Interpretation
drug-resistant *A. baumannii*	-	-	-	-
E. coli ATCC 25922	-	-	0.75	Partially synergistic
P. aeruginosa	-	-	0.27	synergistic
drug-resistant *P. aeruginosa*	-	-	-	-

- representd had no FICI

* FICI, FICI ≤ 0.5, a partially 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

Table 3. MICs, FICs and FICIs of synergism of 3-*epi*-Lupeol combined with Oxytetracycline against *A. baumannii*, *E. coli* ATCC 25922, *P. aeruginosa* and drug-resistant *P. aeruginosa*.

Bacteria	MIC	MIC combination	MIC	MIC Combination	FIC	FIC	FICI*	Interpretation
	Oxy tetracycline	Oxy tetracycline + 3-*epi*-Lupeol	3-*epi*-Lupeol	3-*epi*-Lupeol + Oxytetracycline	Oxy tetracy cline	3-*epi*-Lupeol	3-*epi*-Lupeol	
drug-resistant *A. baumannii*	>2048	-	>2048	-	-	-	-	-
E. coli ATCC 25922	256	128	2048	512	0.50	0.25	0.75	Partially synergistic
P. aeruginosa	64	16	1024	16	0.25	0.02	0.27	synergistic
drug-resistant *P. aeruginosa*	>2048	-	>2048	-	-	-	-	-

- represents having no FICI

* FICI, FICI ≤ 0.5, a partially 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

3.3 Time kill assay

To confirm the synergistic effect of 3-epi-lupeol in combination with Oxytetracycline, a time-kill assay was designed to determine the minimum effective dose for bacterial treatment. The combination of 3-epi-lupeol and Oxytetracycline demonstrated a significant synergistic effect, as shown in Figures 2 and 3. The combination of 3-epi-lupeol with Oxytetracycline exhibited similar antibacterial activity to that of Oxytetracycline alone. When 1/32 MIC of 3-epi-lupeol was mixed with 1/2 MIC of Oxytetracycline, the viability of *E. coli* ATCC 25922 decreased by 84.59±8.19% to 95.10%± 2.3% at 2-8 hours post-inoculation. Similarly, for *P. aeruginosa*, when 1/64 MIC of 3-epi-lupeol was mixed with 1/4 MIC of Oxytetracycline, bacterial growth was inhibited at 2-6 hours after inoculation. The synergistic effectiveness against *E. coli* ATCC 25922 was the most pronounced, showing an increase of 95.10±1.73%, while the effect against *P. aeruginosa* was slightly lower at 58.83±15.56%, observed between 2 to 4 hours after the inoculation of 3-epi-lupeol and Oxytetracycline (Figure 2 and Figure 3). Finally, at 1/32 MIC to 1/64 MIC, 3-epi-lupeol enhanced the bacteriostatic activity of Oxytetracycline, with MIC values ranging from 1/2 MIC to 1/4 MIC.

Considering that 3-epi-lupeol enhanced the effects of Oxytetracycline, the most significant synergistic results against *E. coli* ATCC 25922 and *P. aeruginosa* were confirmed only through the time-kill assay. The results indicated that the combination of 3-epi-lupeol and Oxytetracycline increased the antibacterial activity of Oxytetracycline. In our study, 6.82 and 218.1 μg/mL of 3-epi-lupeol, combined with 6.82 μg/mL (1/4 MIC) and 56.9 μg/mL (1/2 MIC) of Oxytetracycline, reduced the colony-forming units of *P. aeruginosa* and *E. coli* ATCC 25922 by approximately 1.5 $\log_{10}$, respectively. A similar result was reported by Wang *et al*. (2016), who found that the combination of 8 μg/mL ursolic acid with 2 μg/mL Oxytetracycline reduced colony-forming units of gram-positive bacteria, *B. cereus* and *S. aureus*, by more than 2 $\log_{10}$. However, our data contrasts with those reported by Zacchino *et al*. (2017), who found that oleanic acid, a triterpenoid derivative, in combination with aminoglycosides (gentamicin and kanamycin), exhibited synergism against *A. baumannii*. Our results indicated that the combination of 3-epi-lupeol and Oxytetracycline had no synergistic effect against *A. baumannii*. However, our data confirmed that adding 1/32 MIC to 1/64 MIC of 3-epi-lupeol in combination with Oxytetracycline improved the bacteriostatic activity of

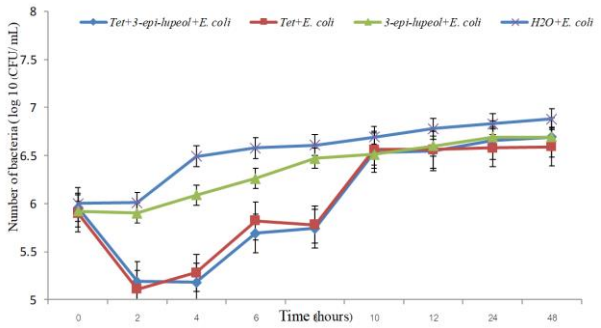

Figure 2. Time kill curve of 1/32 MIC 3-*epi*-lupeol in combination with 1/2 MIC Oxytetracycline against *E. coli* ATCC 25922

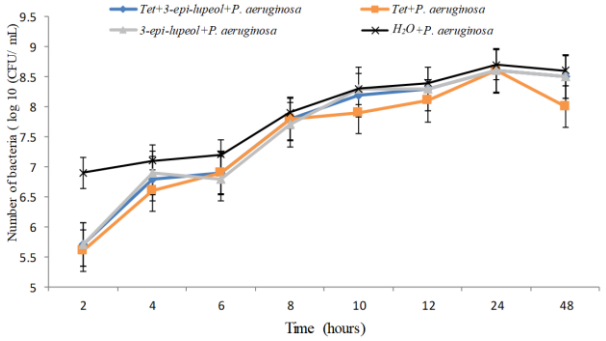

Figure 3. Time kill curve of 1/64 MIC 3-*epi*-lupeol in combination with 1/4 MIC tetracycline against *P. aeruginosa*

Oxytetracycline and reduced the required dose of Oxytetracycline by half compared to using it alone. Additionally, using 3-epi-lupeol by itself may be as effective as some antibiotics. The mechanisms of action of triterpenoids and their derivatives will need further investigation.

3.4 Antibiofilm assay

Chronic infections are often associated with bacterial biofilm formation on medical devices, such as central venous catheters and artificial heart valves (Jamal *et al*., 2018). Searching for new natural products in combination with antibiotics may be a crucial first step in controlling and reducing bacterial biofilm formation, limiting bacterial growth before dissemination to vital organs, and addressing the issue of antibacterial resistance. Biofilm formation by drug-resistant bacteria, such as *P. aeruginosa* and *A. baumannii*, presents a significant challenge in efforts to eradicate biofilm formation. The results of the antibiofilm assay are shown in Figure 4. The antibiofilm activities of 3-epi-lupeol against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and drug-resistant *A. baumannii* were statistically significant ($p \leq 0.05$). The results also indicated that 1,024 μM (equivalent to 436.2 μg/mL) of 3-epi-lupeol exhibited antibiofilm activity against *E. coli* ATCC 25922, *A. baumannii*, and *P. aeruginosa* ATCC 27853 at 2-12 hours after treatment.

The highest inhibitory efficacy against biofilm formation by *E. coli* ATCC 25922, *P. aeruginosa*, and *A. baumannii* was 63.94%, 65.90%, and 72.05%, respectively, at 12 hours. Compared to the antibiofilm activity of 3-epi-lupeol,

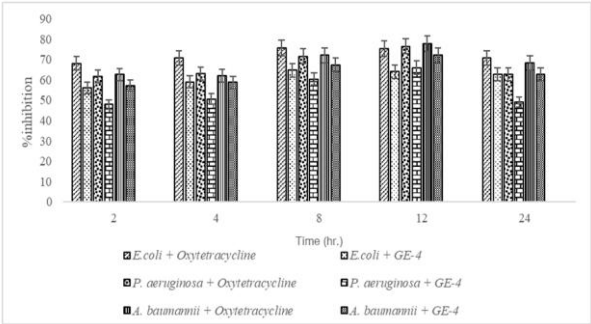

Figure 4. Antibiofilm activity of 1,024 µM 3-*epi*-lupeol (GE-4) against drug resistant *A. baumannii*, *E. coli* and *P. aeruginosa*, compared to antibiofilm activity of 173.8 µM Oxytetracycline

Oxytetracycline exhibited significantly higher antibiofilm activity ($p \leq 0.05$). As shown in Figure 4, 3-epi-lupeol effectively inhibited biofilm formation by the gram-negative bacteria *E. coli*, drug-resistant *A. baumannii*, and *P. aeruginosa*. The antibiofilm activity of 3-epi-lupeol is consistent with the antibiofilm activities of betulinic acid and ursolic acid against *Enterococcus faecalis*, *S. aureus*, and *S. epidermidis* (Silva *et al*., 2019).

The proposed mechanism of lupane suggests that it may inhibit the synthesis of proteins associated with planktonic bacterial formation (Silva *et al*., 2019). This result is consistent with the study by Evaristo *et al*. (2014), who found that the triterpene 3β,6β,16β-trihydroxylup-20(29)-ene from *Combretum leprosum* Mart. inhibited the formation of planktonic cells by *Streptococcus mutans* and *S. mitis*, but did not inhibit biofilm formation by *P. aeruginosa* (Evaristo *et al*., 2014). Interestingly, this result showed that 3-epi-lupeol exhibited strong antibiofilm activity against drug-resistant *A. baumannii*. As a result, 3-epi-lupeol could be considered a novel compound derived from natural products with antimicrobial properties for the treatment of bacterial infections. However, further work is needed to examine cytotoxicity and the mechanisms of antibacterial action. This study opens the door to the possibility that 3-epi-lupeol, in combination with Oxytetracycline, may enhance the antibacterial and antibiofilm activities of conventional antibiotics against certain opportunistic bacteria.

4. Conclusions

The results of this study revealed that 3-epi-lupeol exhibited antibacterial and antibiofilm activities against some opportunistic and drug-resistant bacteria. Additionally, 3-epi-lupeol enhanced the antibacterial activity of the conventional antibiotic Oxytetracycline against certain bacteria. However, 3-epi-lupeol did not show any synergistic effect with Ampicillin. The single use of 3-epi-lupeol and its combination with Oxytetracycline may offer an alternative approach to enhance the antibacterial activity of conventional antibiotics.

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