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Original Research Article

Antibacterial and Antibiofilm Activities of Lupanes from *Glochidion daltonii* against some Opportunistic Bacteria

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ABSTRACT

The research proposed natural compounds, derived from Khrai, *Glochidion daltonii* (Muell. Arg (Kurz). The pure compounds from *G. daltonii* were screened for antibacterial activity and compared to conventional antibiotics, ampicillin and tetracycline against some opportunistic bacteria. Then, antibiofilm activity of lup-20 (29)-ene-3α, 23-diol was determined. The results showed that five lupanes; 3-epi-Lupeol (GE-4), Glochidone (GE-11), Glochidonol (GE-12), Lup-20)29(-ene-3α, 23-diol (GE-14) and Glochidiol (GE-15) had antibacterial activities and inhibited the growth of some opportunistic bacteria at MICs ranging from 64-2048 µM. Compared to two antibiotics, all five lupanes were more active than ampicillin, such as active against *P. aeruginosa*, but were less efficacious than tetracycline. In addition, the antibiofilm activity of some lupanes was expressed. GE-14 and GE-15 showed antibiofilm activity against tested bacteria, and %inhibition ranged between 10-50%. Compared to the antibiofilm activity of GE-14 and GE-15, the antibiofilm activity of tetracycline showed better antibiofilm activity than the antibiofilm activities of GE-14 and GE-15. So far, lupanes from *G. daltonii* could be developed as a novel antibacterial agent.

INTRODUCTION

Antibiotic resistance caused by bacteria is one of the serious problem and has an impact on the treatment of infectious diseases. So far, the incidence of infection caused by drug resistant bacteria is constantly increasing (Reygaert, 2018). Thus, an increase in bacterial infection caused by drug resistant bacteria may contributed to high mortality and treatment costs. It has been estimated that 700,000 deaths in hospitals are associated with infection of drug resistant bacteria. If the problem of antibiotic resistance is not resolved by the year 2050, antimicrobial resistance will cause 10 million deaths which will be higher than the deaths caused by cancer and diabetes (Majumder *et al*., 2020). Medicinal plants have many phytochemical components of which function in many modes of action, especially maintenance and promotion immunity of human (Subramani *et al*., 2017). Thus, finding novel antibacterial agents derived from medicinal plants may a priority work of scientists.

Glochidion daltonii is a monoecious shrub tree found in the forest of China, Thailand, Taiwan and Vietnam. All parts of *G. daltonii*, especially roots and leaves are used as folk medicine for

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curing uticaria, mastitis, menorrhagia, dysentery, enteritis, toothache (Chaturvedi, Bhui, and Shukla, 2008). The lupanes, a type of triterpene, derived from *G. sphaerogynum* and *G. eriocarpum* have been identified and reported anticancer activity (Puapairoj, *et al.* 2005). In the meanwhile, lupanes from many plants were proposed as program cell death disruption and promote intrinsic pathway of apoptosis against Hepatic cancer, Colorectal cancer, Breast cancer (Cháirez-Ramírez *et al.*, 2016). Some report revealed antimicrobial activities of the four lupanes form *Drypetes inaequalis* that lupanes inhibited the growth of *Staphylococcus. aureus*, *Escherichia coli*, *Klebsiella pnuemoniae*, *Salmonella typhi, Shigella dysenteriae, and Pseudomonas aeruginosa* (Awanchiri *et al.*, 2009). In the current study, lupanes and terpenes have commonly reported the antibacterial activity and have and action like antibiotics against some antibiotic resistant pathogen such as oleanic acid as a strong antibiotic property against *A. baumannii* and carvone as an antibacterial agent against *E. coli* and *P. aeruginosa*. (Mahizan *et al.*, 2019).

Biofilm formation is an accumulation of bacteria that attaches to high moisture surface and then bacteria produce matrix like polymers consisted of carbohydrates, proteins and nucleic acids. Biofilm formation leads to prevention of bacterial colonization from eradication by antibacterial agents (Rabin *et al.*, 2015). Currently, many researchers have reported of pure substances and herbal extracts that can inhibit biofilm formation of bacteria such as trans cinnamaldehyde from cinnamon bark, terpenes from essential oils derived from lemongrass and clove (Gupta *et al.,* 2017; Silva *et al.,* 2019). Also, terpinen-4-ol from *Melaleuca alternifolia* inhibited biofilm formation of *S. aureus* (Cordeiro *et al.*, 2020). Some triterpenoids from *Sarcochlamys pulcherrima* (Roxb.) Gaud have potentially antibiofilm activity against *P. aeruginosa* (Ghosh *et al.*, 2020).

According to decreasing number of multidrug resistant bacteria from colonization and inhibiting the growth of multidrug resistant bacteria, this research was to examine the antimicrobial and antibiofilm activities of five lupanes from *G. daltonii*. This work may propose that some derivatives from medicinal plants like *G. daltonii* could be a supplement product that can replace antibiotics with lupanes for treatment of bacterial infection.

MATERIALS AND METHODS

Plant materials

The five derivatives of lupanes from roots and stems of *G. daltonii* (voucher speciemens No. BKF147875). The 1 kg of roots and stems of *G. daltonii* were extracted by 100% hexane. The crude extract was harvested and then was separated by incremental gradient elution by column chromatography. The gradient elution was started from 100 % hexane to100 % ethyl acetate, and finished by 100% methanol. The five crystals were formed, and the pure compounds were characterized as lupanes by ^{1}H NMR, ^{13}C NMR, DEPT-90 and DEPT-135 spectrum (Puapairoj *et al.,* 2005). The molecular weights of all lupanes are shown in Table 1

Bacterial strains

E. coli ATCC 25922 were kindly provided from Department of Microbiology, Burapha Universiy, Chonburi, Thailand. The two resistant strains, *A. baumannii*, resisted to aminoglycoside, β-lactam and quinolone and *P. aeruginosa* Code 1-375/04-2013, resisted to aminoglycoside, β-lactam, carbapenem and quinolone were provided from Chonburi Hospital, Chonburi Province, Thailand. All strains were cultured in nutrient agar (Difco, U.S.A.) and characterized by API 20 NE System

Table 1. Molecular weights of five lupanes extracted from roots and stems of *G. daltonii*

Lupanes from *Glochidion daltonii*	Molecular weights
3-Epi-lupeol (GE-4)	426
Glochidone (GE-11)	442
Glochidonol (GE-12)	440
Lup-20(29)-en-3α, 23-diol (GE-14)	442
Glochidiol (GE-15)	442

Determination Minimum Inhibition Concentration (MIC)

Broth microdilution susceptibility assay was designed along well with the method of CLSI (2017). Briefly, 2048 µM of five lupanes was diluted using a serial dilution range 8 µM - 2,048 µM by 100 µl MHB in a microplate (VersaMax, U.S.A.). Then, 100 µl of 1 x10^8 CFU/ml of bacterial culture was added and incubated at 37°C for 18-24 h. The viable count of bacteria was measured by optical density at 610 nm (O.D. 610) by a microplate reader (VersaMax, U.S.A.). The standard antibiotics of 8 µM - 2,048 µM; ampicillin and tetracycline were tested as positive controls, and deionized distilled water was the negative control. Each treatment was performed in triplicate. Finally, the lowest concentration that reduces 100% of O.D. of microorganism was recorded as MIC.

Assay of Antibiofilm activity

The antibiofilm activity was evaluated by microplate assay, modified from Song *et al.* (2019). Briefly, 100 µl aliquot of lupanes or positive control (tetracycline) was added in flat-bottomed 96-well microplates. Then, 100 µl of 1x 10^6 CFU/mL of bacterial culture, and 100 µL of Tryptic Soy broth (TSB, Difco, U.S.A.) were applied to microplates. The microplates were incubated for 2, 4, 8, 12 and 24 h (without shaking). At an interval time, each well of sample (contained adherent biofilm of bacteria) was stained by 1% crystal violet for 15 minutes and unstained bacteria was washed out by phosphate-buffer saline. The bacterial biofilm was fixed with 200 µl 100% methanol for 15 minutes and air dried. The stain of bacterial biofilm was solubilized by 150 µL of 33% glacial acetic acid. Then, the soluble biofilm of bacteria was determined using a microplate reader VersaMax (U.S.A.) at an optical density of 600 nm. The absorbance and percentage inhibition of biofilm was determined by equation 1

$$\text{Percentage inhibition} = \frac{(\text{OD negative control} - \text{OD experiment})}{\text{OD negative control}} \times 100 \qquad (1)$$

Statistical analysis

The optical densities of bacterial growth and antibiofilm test were analyzed by two-way analysis of variance (ANOVA) and depended on variables, treatment and times. The differences among means were analyzed by Duncan's multiple range tests using Minitab software version 17 (Minitab Pty Ltd, Sydney, Australia) with a significance at $p \leq 0.05$

RESULTS AND DISCUSSION

Determination MICs of Lupane

The five lupanes *from G. daltonii* showed effective antibacterial activities against opportunistic bacteria and drug resistant bacteria; *A. baumannii, E. coli* ATCC 25922 and *P. aeruginosa* (Table 2). Lup-20(29)-en-3α, 23-diol (GE-14) was the most lupane that was effective against *E. coli* ATCC 25922 and *P. aeruginosa*. All five lupanes showed MICs ranging between 64-2048 µM. Moreover, antibacterial activity of tetracycline was the most efficacious against *E. coli* ATCC 25922 and *P. aeruginosa* except *A. baumannii*. However, ampicillin had no antibacterial activity against tested bacteria. Antibacterial activities of lupanes, ampicillin and tetracycline against all opportunistic bacteria were significance different ($P \leq 0.05$). In these results, MICs of five lupanes were indicated the less antibacterial activity than MICs of lupeol and betulinic acid (Mahizan *et al*., 2019). The five lupanes from this study are triterpene derivatives, and somehow lupanes may play a role on bacterial efflux pumps and disruption cell membrane of bacteria (Mahizan *et al*., 2019). Additionally, there was a report expressed that terpene may interfere ion transport system and disrupt phospholipid bilayer of bacterial cell membrane (Gupta & Birdi, 2017).

Antibiofilm activity of lupanes

Some of antibiofilm activities of GE-14 and GE-15 are shown in Figures 1 and 2. From the results of the study, it was found that GE-14 and GE-15 showed low antibiofilm activities, less than 50% inhibition against *A. baumannii*, *E. coli* ATCC 25922 and *P. aeruginosa*, and %inhibition ranged between 12.31- 48.65%. Compared to the antibiofilm activity of tetracycline, antibiofilm activity of GE-14 and GE-15 showed less antibiofilm activity and %inhibitions of tetracycline ranged between 50.06 - 67.66%. *G. daltonii* may contained the same groups of lupanes as in *Combretum leprosum* so that lupanes from *G. daltonii* could inhibit planktonic growth and biofilm formation (Evaristo *et al*., 2014). GE-14 and GE-15 isolated from *G. daltonii* are confirmed as triterpene as the same class of 3β,6β,16β-trihydroxylup-20(29)-ene found in *Combretum leprosum* (Evaristo *et al*., 2014). Thus, GE-14 and GE-15 may inhibit formation of planktonic cell of bacteria, and this mechanism will be verified.

Table 2. Antibacterial activities of five lupanes.

Bacteria	MIC (µM)						
	GE-4	GE-11	GE-12	GE-14	GE15	Ampicillin	Tetracycline
A. baumannii	>2048	>2048	>2048	>2048	>2048	>2048	>2048
E. coli ATCC 25922	2048	>2048	>2048	512	2048	>2048	16
P. aeruginosa	1024	2048	64	512	2048	>2048	64

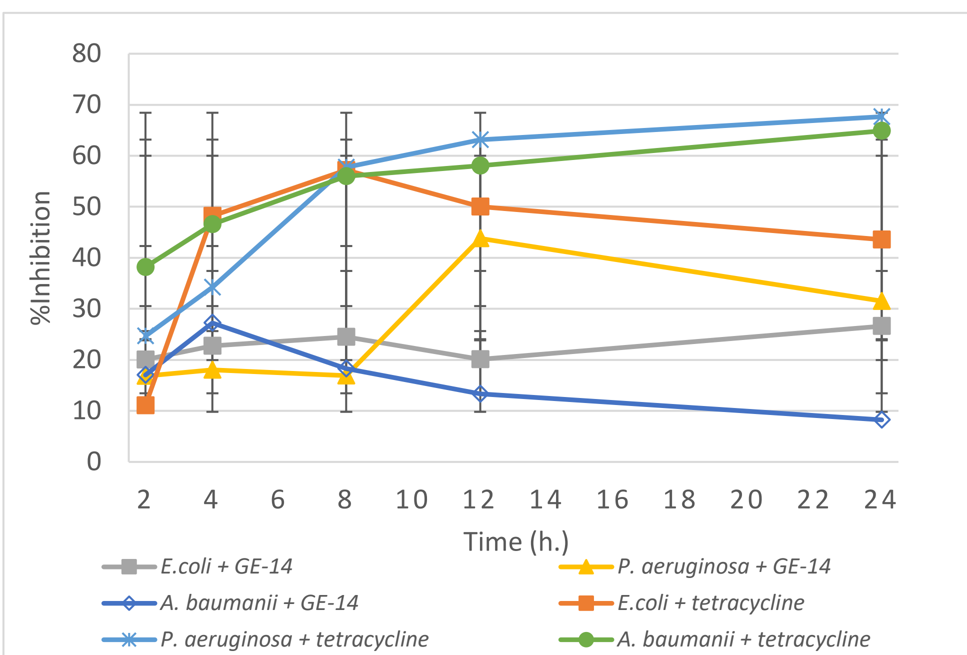

Figure 1. The antibiofilm activity of GE-14, compared to antibiofilm activity of tetracycline against some opportunistic and drug resistant bacteria.

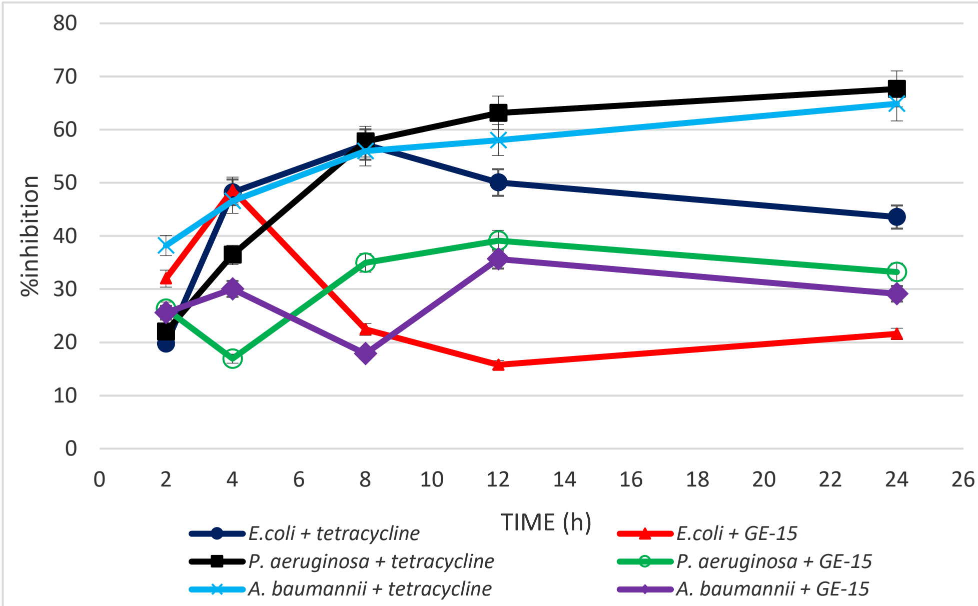

Figure 2. The antibiofilm activity of GE-15, compared to antibiofilm activity of tetracycline against some opportunistic and drug resistant bacteria.

Conclusions

Five derivatives of lupanes from roots and stems of *G. daltonii* showed antibacterial activity and antibiofilm activity against opportunistic and drug resistant bacteria; *A. baumannii*, *E. coli* ATCC 25922, and *P. aeruginosa*. Our research showed some successful inhibitors for eradicating biofilm formation of drug resistant bacteria. In application, lupanes from *G. daltonii* were the active compounds as some antibiotics of which are low cost and reduce side effect of bacterial infection.

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