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Antibacterial Activity of *Tylophora tenuis* Methanol Extract on *Staphylococcus aureus*

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ABSTRACT

Tylophora tenuis is a species belonging to *Tylophora* genus, Asclepiadaceae. This study aimed to identify the plant species and evaluate the antibacterial activity of *T. tenuis* methanol extract on *Staphylococcus aureus*. The plant was extracted with methanol using soxhlet apparatus. The antibacterial property of the extract was evaluated using disk diffusion method. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) were determined by broth dilution method. The results showed strong antibacterial activity of *T. tenuis* at 500 mg/mL (10 mg/paper disk) with the zone inhibition diameter was 18.33 ± 0.58 (mm). The MIC and MBC of the extract against *S. aureus* were 62.6 mg/mL. In conclusion, the use of methanol extract from *T. tenuis* could inhibit the growth of *S. aureus in vitro*.

Keywords: *Tylophora tenuis*, bacteria, inhibition, extract, soxhlet

INTRODUCTION

The Antimicrobial resistant (AMR) bacteria have infected and caused death to million people worldwide. In 2019, there were 4.95 million deaths related to antibiotic resistance infection. The leading pathogens causing deaths were estimated including Escherichia coli, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Among them, methicillin-resistant *S. aureus* caused more than 100,000 deaths associated with AMR (1). *S. aureus* are one of the most common microbial infections in humans. They are located on the skin and mucous membranes of the body's canal and organs. *S. aureus* are the major agent of multiple infectious diseases such as bacteremia, infective endocarditis, skin and soft tissue infections, osteomyelitis, and pulmonary infections. The prevention of *S. aureus* infection is still a challenge due to its antibiotic-resistant ability, especially with methicillin-resistant strains (MRSA) (2). Therefore, developing a new treatment to fight AMR bacteria are crucial to scientists, and natural product with antibacterial activities are promising sources (3).

Genus *Tylophora* (Asclepiadaceae family) is widely developed from Australia, Asia to Africa. *T. indica* is a common plant in the genus with multiple uses in traditional medicine and scientific publications. Phytochemical constituents from *Tylophora* species have been isolated. For instance, their alkaloids were determined such as tylophorine, tylophorinidine, septicine, and tylophorine, as well as non-alkaloidal structures such as quercetin, tannins, tetratriacontanol, α- and β-amyrins, octaosanyl octacosane (4). Many species in this genus have widely been used in folk medicine in tropical and subtropical regions. These plants possess a variety of activities such as anti-inflammatory (*T. barbata*), antiallergic (*T. asthinatica*, *T. ovata*), asthma, dermatitis, (*T. indica*),

hypoglycemic (*T. hirsuta*), wound healing (*T. perrottetiana*) (4), antitumor, antioxidant and antimicrobial activities (T. indica) (4-7). *Tylophora tenuis* Blume. usually perennial twining, less often herbaceous, and/or erect. Leaves are opposite. Small flowers are pinkish to brownish, long pedicel, and capillary (8). *T. tenuis* was demonstrated to have antibacterial effect on B. cereus (9), however, there is still insufficient information about the biological activity of this species. Hence, this study was performed to investigate the antibacterial effect of *T. tenuis* on *S. aureus in vitro*.

MATERIALS & METHODS

Plant Identification

DNA from leaves of *T. tenuis* were extracted using CTAB protocol, followed by the amplification of Internal transcribed spacer (ITS) region with primer sequences: ITS1: 5' TCCGTAGGTGAACCTGCGG 3' (Forward)/ ITS4: 5' TCCTCCGCTTATTGATATGC 3' (Reverse). After sequencing by Sanger sequencing, the query nucleotide sequence was compared with data of sequence in the Basic local alignment search tool (BLAST) to identify the match species.

Plant Extraction

Leaves and stems of *T. tenuis* were collected from the local area in Tra Vinh province, Viet Nam. Dust was removed and materials were dried under 50 ^{o}C until a constant weight using the oven-dry. Dried materials were ground with a blender. Plant material was extracted in hot methanol using soxhlet apparatus. Then, the solvent was removed under reduced pressure by using a rotary evaporator and water bath. The extract was kept at -20 ^{o}C for further studies.

Bacteria Species

S. aureus (VTCC 10658) was purchased from the Institue of Microbiology and Biotechnology, Viet Nam. The bacteria were pre-cultured on MHA (Muller Hinton Agar) or inoculated in MHB (Muller Hinton Broth) before doing experiments.

Antibacterial assay

Disk diffusion methods: the extract was redissolved in 2% DMSO to achieve different concentrations of 62.5, 125, 250, and 500 mg/mL. Add 20 µL of each extract concentration or 2% DMSO control to the sterilized paper disk (6 mm diameter). Gentamicin 10 µg/disk was employed as a positive control antibiotic.

One hundred (µL) of overnight inoculated *S. aureus* (1.5x106 CFU/mL) was spread on the surface of MHA plate. Then, extract loaded-paper disks, 2% DMSO, and gentamicin were placed on the surface of MHA. The plates were stood in the fridge for 2 h before incubating at 37 ^{o}C. The zone inhibitions were measured after 24 h.

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC):

MIC and MBC were determined using the broth dilution method as described before with slight modification [11]. Different concentrations of extract (0 – 500 mg/mL) or gentamicin 1 mg/mL were added to 1 mL of MHB media, and mixed thoroughly. 100 µL of bacterial suspension (5 x 105 CFU/mL) was transferred to the tubes in the series and mixed well before incubating at 37 °C for 24 h. The antibiotic agent–free medium was carried out as bacteria control. The MIC value was the lowest concentration of extract showing no visual growth of bacteria.

For MBC detection, aliquoted 100 µL of the solution from the series tube from MIC to distinguish MHA plates, and spread the bacteria on MHA plates. The developing colonies were counted after 24 h cultured in the 37 ^{o}C incubator. The MBC was defined as the lowest concentration of extract which is a complete inhibition of bacteria growth.

STATISTICAL ANALYSIS

All the experiments were performed in triplicate. The results were expressed as the mean ± SD (n=3). The comparison was made by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test using Sigmastat

software. The significant difference between groups was confirmed when $P < 0.05$.

RESULT

Plant Identification

The amplification and sequencing results using the primer of ITS1/ITS4 resulted in 627 nucleotides length. The highest match of molecular identification was 99.84% with species *T. tenuis* (**Figure 1**). Combining with the morphology of the specimen, we concluded that the plant used in this experiment was *T. tenuis*, family Asclepiadaceae.

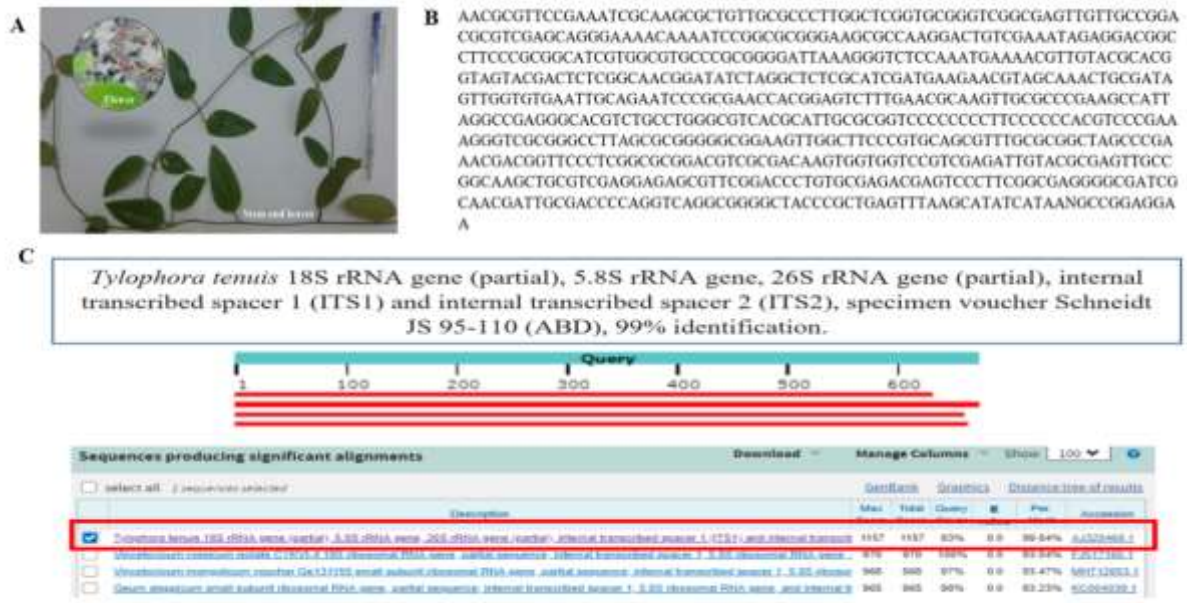

Figure 1. Plant identification of *T. tenuis*
A. Morphology of *T. tenuis*, B. The ITS region sequence by Sanger sequencing, C. Matched with nucleotide sequence in BLAST

Antibacterial activity of *T. tenuis*

T. tenuis methanol extract showed strong inhibition activity against the growth of *S. aureus* (**Figure 2A**). The diameter of the inhibition zone increased when the bacteria were exposed to a higher concentration of extract (**Table 1**). In this experiment, the extract exhibited the strongest antibacterial effect at 500 mg/mL with the width of the inhibition zone being 18.33 ± 0.58 (mm). Followed by the extract at concentrations of 250 mg/mL (17.33 ± 0.58 mm), 125 mg/mL (14.33 ± 0.58 mm), and 62.5 mg/mL (11.33 ± 0.58 mm). These results showed dose-dependent activity of extract on *S. aureus* growth inhibition. There was no statistical difference found when using extract at two low concentrations as well as when using two high concentrations ($P>0.05$). The methanol extract of *T. tenuis* inhibition potential on *S. aureus* is significantly weaker than standard gentamicin control (28.00 ± 0.00 mm, $P<0.05$) at all used concentrations.

Table 1. The antibacterial activity of *T. tenuis* methanol extract on *S. aureus*

Extract (mg/mL)	Used volume (µL)	Amount per paper disk (mg)	Inhibition zone diameter(mm)
2% DMSO	20	0	0.00 ± 0.00^{a}
62.6	20	1.25	11.33 ± 0.58^{b}
125	20	2.5	14.33 ± 0.58^{b}
250	20	5	17.33 ± 0.58^{c}
500	20	10	18.33 ± 0.58^{c}
Gentamicin	-	10µg	28.00 ± 0.58^{d}

The data were expressed as Mean ± SD (n=3).
The different alphabet represents the significant difference between groups by One-way ANOVA test on rank followed by SNK test ($P<0.05$).

Minimum inhibition concentration and minimum bactericidal concentration

The minimum inhibition concentration and minimum bactericidal concentration of *T. tenuis* extract on *S. aureus* were determined using the broth dilution method (**Figure 2B**). MIC value is the lowest concentration of antimicrobial agents that inhibit the visible growth of bacteria after overnight incubation. MBC is the lowest concentration of antibacterial agents necessary to kill bacteria (10). In this study, the extract showed antibacterial activity in *S. aureus* with MIC and MBC values were 62.5 mg/mL (**Figure 2B**).

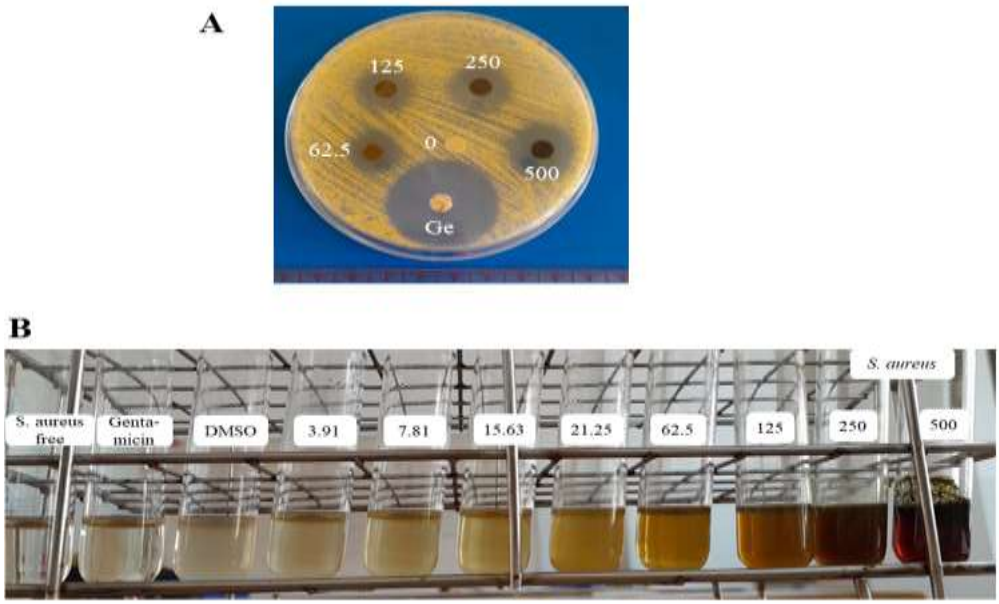

Figure 2. Antibacterial activity of *T. tenuis*
A. Zone inhibition (mm), B. Serial dilution broth assay

Table 2 shows the percentage of inhibition of *T. tenuis* extract on the viability of *S. aureus*. At 1.95 mg/mL, the extract could kill almost 50% *S. aureus* after 24 h exposed. The effect was doubled when the concentration increased twice. Especially, at 62.5 mg/mL, the inhibition reached 99.98%. Therefore, 62.5 mg/mL was also determined as the MBC value of the extract. The inhibition of *T. tenuis* extract clearly showed dose-dependent activity (P<0.05).

Table 2. Percent inhibiton of T. tenuis (mg/mL) on the viability of S. aureus

Extract concentration	Genta-micin	250	125	62.5	31.25	15.63	7.81	3.91	1.95	DMSO
% inhibition on S. aureus	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a	99.98 ± 0.00a	99.84 ± 0.00a	98.18 ± 0.10b	90.13 ± 0.49c	86.09 ± 0.43d	48.86 ± 1.57e	0.00 ± 0.00f

The data were expressed as Mean ± SD (n=3.
Different alphabet represents the significant difference between groups by One-way ANOVA test followed by SNK test (P<0.05)

DISCUSSION

The rapid development of antibiotic resistance bacteria required more new and novel drugs against those resistant pathogens. Medicinal plants have been considered a huge source of antibacterial agents. Natural antibiotics from plants possess different modes of action and become an interesting field for scientists (11). Natural compounds might play the main role in antimicrobial activities. The antibacterial action mechanisms of phytoconstituents are proven. For instance, flavonoids can inhibit the synthesis of nucleic acids, disturb the function of the cytoplasmic membrane, interfere with membrane porins function, and kill bacteria (12). The action of saponins can be binding

to steroids on the surface of the endoplasmic reticulum disrupting the biofilm system of the cell and changing the structure of macromolecules in the cell (13). Polyphenols are well-known for their antioxidant, anti-inflammatory, and antimicrobial properties (14). Phytochemical constituents of methanol extract of *T. tenuis* were reported earlier. According to Ho et al, the extract contains polyphenols, alkaloids, flavonoids, saponins, and triterpenoids (15). Hence, the mechanism underlying the antibacterial activity of *T. tenuis* on *S. aureus* might be due to the combined action of a variety of phytochemical compounds.

Recently, the antibacterial potential of plants belonging to the genus *Tylophora* has been demonstrated. For instance, *T. indica* extract shows broad activity against both Gram-negative and Gram-positive pathogens. The crude ethanolic extract of *T. indica* leaves can depress bacterial and fungal development including *K. pneumoniae*, *S. aureus*, *S. typhi*, *P. vulgaris*, *E coli*, *A. niger*, and *A. fumigatus* (16). Aqueous extract of mixed leaves and stem of *T. indica* also inhibit *E. coli* and P. *aeruginosa* growth. When used along with tetracyclin, the extract promotes antibacterial effect of tetracylin (17). The presence of steroids, cardiac glycosides, saponins, phenolics, and alkaloids may respond to the antibacterial action which is found in *T. tenuis* methanol extract.

In this study, MIC and MBC of extract were determined. Both MIC and MBC have the same value of 62.5 mg/mL. The results were a little high but in a range with the effect of *T. indica*. The plant has antibacterial effect on *Enterococcus faecalis* with MIC and MBC values were 40 and 180 mg/mL (18). In this study, the MIC and MBC values of *T. tenuis* on *S. aureus* is comparable to those action of *B. cereus*. MIC value of *S. aureus* (62.5 mg/mL) is greater than the MIC value measured of B. cereus (31.25 mg/mL). However, the MBC recorded (62.5 mg/mL) is much lower than that of *B. cereus* (125 mg/mL) (9). MIC and MBC values from this study were a little high due to the use of crude extract. Previous studies show the high MIC and MBC value of crude extract in antibacterial activity. The antibacterial activity of *Tanacetum vulgare* crude extract tested on *E. coli*, *S. agalactiae*, *T. vulgare*, *Streptococci*, and *Serratia liquefaciens* is range from 53.9 to 125.9 mg/mL (19) or the antibacterial ability of *Bixa orellana* stem and leaves extract on *B. cereus* were with MIC = 34.71 and 14.11 mg/mL, respectively (20). Therefore, *T. tenuis* has the potential to be an antibacterial candidate from nature.

CONCLUSION

In conclusion, the present study indicates that methanolic extract of *T. tenuis* possessed an antibacterial effect on *S. aureus*, a Gram-positive bacteria. The species of plant is *T. tenuis* which is confirmed by DNA barcoding. The extract showed a strong activity target to the growth of *S. aureus* at high concentrations but lower activity of gentamicin. The results suggest the using potential of *T. tenuis* as an alternative anti-*S. aureus* agents for infectious treatment and the toxic of the plant need to be clarified.

Declaration by Authors
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Conflict of Interest: The authors declare no conflict of interest.

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