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The Inhibition of *Tylophora tenuis* Methanol Extract on *Bacillus cereus*

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Abstract: This study aims to investigate the inhibitory activity of *Tylophora tenuis* on Bacillus cereus, a Gram-positive bacterium causing emetic and diarrhoeal syndromes. Leaves and stem of *T. tenuis* were collected from the local area, dried and ground before being extracted with methanol using soxhlet apparatus. The extract was screened for its phytochemical contents and antibacterial activity on *B. cereus* through disk diffusion method. MIC and MBC were determined by broth culture method and plate counting. The results showed that *T. tenuis* contains alkaloids, flavonoids, polyphenols, saponins, and triterpenoids. The extract exhibited the bacterial inhibition zone up to 24.67 ± 0.58 mm at 20 mg per paper disc. The values of MIC and MBC were 31.25 and 125 µg/mL, respectively. From this study, we concluded that the methanol of extract of T. tunius possesses antibacterial inhibition effect on *B. cereus* due to its phytochemical components.

IndexTerms*: *Bacillus cereus, Tylophora tenuis, antibacterial, phytochemicals, extract

I. INTRODUCTION

Bacillus cereus is a Gram-positive bacterium widely distributed in the environment such as soil and various contaminant food such as vegetables, milk, and meats. By consuming *B. cereus*-contaminated foods, humans are at risk of gastrointestinal illness due to the bacterial toxin production of hemolysins, phospholipases, and pore-forming enterotoxins. These toxins can lead to diarrheal syndrome with nausea and vomiting, the non-hemolytic enterotoxin from *B. cereus* can trigger programmed cell death by activating inflammatory caspases of infected tissues [1]. For non-gastrointestinal diseases, *B. cereus* infection can also cause serious and life-threatening conditions including bacteremia, meningitis in children, endocarditis, endophthalmitis, and pneumonia. This bacterium is reported to associate with diabetes, traumatic injuries, and immunocompromised states conditions [2].

In the increasing of phytotherapy to prevent and cure illness, there are abundant studies on antibacterial capacity of medicinal plants. Although *B. cereus* species show sensitivity to vancomycin, gentamicin, and imipenem, some exhibit resistance to cephalosporins, clindamycin, and levofloxacin [3]. Many plants reveal antibacterial activity on *B. cereus* such as clove, cinnamon, curcumin, black pepper, ginger, and saffron. Clove water extract and all ethanol extracts show anti-*B. cereus* activity with the clear zone diameter reaching 25 mm (clove extract) and 30 mm (cinnamon extract) [4]. When exposed to the ethanol extract of cinnamon bark and fingerroot at a concentration of 5% w/v, *B. cereus* spores are killed within 1 h [5]. The two novel cassane diterpenoids isolated from *Caesaplinia pulcherrima* possess powerful activity against B. cereus with MIC values of 3.13 µM by interfering with bacterial cell membrane proteins and membrane permeability causing cell damage or death [6].

Tylophora tenuis Blume (**Fig. 1**) is a member of the genus *Tylophora*, Asclepiadaceae family. Plants are usually perennial twining, less often herbaceous, and/or erect. Leaves are paired and opposite arranged at nodes. The plant has small pinkish to brownish flower, long pedicel, and capillary [7] (Figure 1). Many species in this genus have widely been used in folk medicine in the tropical and subtropical regions. These plants possess a variety of activities such as antiinflammatory (*T. barbata*), antiallergic (*T. asthinatica*, *T. ovata*), asthma, dermatitis, cough (*T. indica*), hypoglycemic (*T. hirsuta*), wound healing (*T. perrottetiana*) [8], antitumor (*T. indica*) [9], antioxidant and antimicrobial activities (*T. indica*) [6, 8, 10]. However, there is no report of antibacterial effect of *T. tenuis* found as well as other biological activities. Hence, this study was conducted to investigate the antibacterial effect of *T. tenuis* on *B. cereus* and screened its phytochemical components.

II. MATERIALS AND METHODS

2.1 Plant material and extraction

Tylophora tenuis was collected from the local area in Tra Vinh province, Viet Nam. The flowers and root system were removed. Leaves and stems were washed out of dust and dried under 50°C until a constant weight was achieved using the oven-dry. Dried plant parts were ground with a blender. For methanol extract, 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°C for further studies.



Figure 1. *Tylophora tenuis* Blume: stem, leaves and flowers

2.2 Bacteria species

Bacillus cereus (VTCC 11917) 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.

2.3 Phytochemical screening of T. tenuis methanol extract

Phytochemical components of *T. tenuis* methanol extract were determined following the standard method described by the University of Medicine and Pharmacy at Ho Chi Minh City, Viet Nam.

Test for alkaloids: small amount of extract was dissolved in 5% HCL. Alkaloids were detected by adding to 1 mL of sample several drops of Bouchardat reagent in a test tube, the formation of a brown precipitate demonstrated the presence of alkaloids.

Test for flavonoids: small amount of extract was dissolved in ethanol, added a small amount of magnesium powder and 0,5 mL concentrated HCL. The pink or red color indicates the presence of flavonoids.

Test for polyphenols: small amount of extract was dissolved in distilled water above a heated water bath, added 2 – 3 drops of 5% $FeCl_3$ to 2 mL of extract with gently mixed. A dark green or moss-green color indicates the presence of phenolic compounds.

Test for tannins: small amount of extract was dissolved in distilled water above a heated water bath, added 5 drops of gelatin salt to 2 mL of extract with well mixed. The white turbidity form indicates the presence of tannins.

Test for saponins: saponins were indicated by shaking vigorously 10 ml of water-dissolved extract in a test tube. The formation of a layer of stable persistent foam confirms the presence of saponins.

Test for triterpenoids: For triterpenoids, dissolved a small amount of extract in 0.5 mL of acetic anhydride. To this tube, added 1 mL of chloromethane, mixed, and tranferred to a new dried tube following by carefully adding 2 mL of concentrated H2SO4. A reddish-brown color layer formed in the interface layer shows the existence of terpenoids.

2.4 Antibacterial assay

Disk diffusion methods: the extract was redissolved in 2% DMSO to achieve different concentrations of 250, 500, and 1000 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.

For bacterial preparation, 100 µL of overnight inoculated *B. cereus* ($1.5x10^6$ CFU/mL) was spread on the surface of 90 mm petri plate containing MHA. Paper disks loaded with extract, 2% DMSO, and gentamicin were placed on the surface of MHA. After that, plates were stood in the fridge (4-8ºC) to let the extract diffuse out for 2 h before incubating the plate at 37ºC, and measuring the presence or absence of a zone of inhibition around paper disks after 24 h.

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

MIC and MBC were determined using 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, mixed thoroughly. Then, 100 µL of B. cereus suspension ($5 x 10^5$ 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, spread the bacteria on the agar surface, and incubated for 24 h in the 37ºC incubator. The inhibition was determined by counting the number of colonies developing on the plates. The MBC was the lowest concentration of extract which a complete inhibition of bacteria growth.

2.4 Statistical analysis

All the experiments were performed in triplicate. The results were expressed as the mean ± SD. 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 $P < 0.05$.

III. RESULTS AND DISCUSSION

3.1 Phytochemical constituents

Phytochemical screening of methanol extract from *T. tenuis* resulted in the presence of many popular compounds found in medicinal plants (**Table 1**). We confirmed the presence of polyphenols, alkaloids, flavonoids, saponins, and triterpenoids but tannins were absent.

These bioactive components were also found in various species belonging to the genus *Tylophora*. The ethanolic extract of *T. pauciflora* contains alkaloids, terpenoids, steroids, flavonoids, glycosides, tannins, and reducing sugar. Nevertheless, tannins are absent in petroleum ether and water extract [12]. The active compounds such as alkaloids, flavonoids, tannins, and phenols are present the most in the ethanolic extract of *T. subramanii* [13]. *T. indica* is the most common in the genus with numerous publications about biological activities and phytochemical constituents including alkaloids, terpenoids, steroids, flavonoids, phenols, glycosides, carbohydrates, amino acids, and saponins. Evidence shows that tannin is absent in methanol extract of *T. indica* [14]. The most of biological benefits of medicinal plants occur as a result of bioactive component activities. Therefore, *T.*

tenuis has the potential to be applied for health protection as an agent for antioxidant, antibacterial, anticancer, or antidiabetic therapy.

Table 1. Phytochemical screening of methanol extract of *T. tenuis*

Phytochemical compounds	Result
Polyphenols	Positive
Alkaloids	Positive
Flavonoids	Positive
Saponins	Positive
Steroid	Positive
Tannins	Negative
Triterpenoids	Positive

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3.2 Antibacterial activity of *T. tenuis*

By agar disk diffusion method, *T. tenuis* extract showed strong inhibition activity against the growth of *B. cereus* (**Fig. 2A**). The diameter of the inhibition zone increased when the bacteria were exposed to a higher concentration of extract (**Table 2**). Among tested concentrations, the extract revealed the strongest antibacterial effect at 1000 mg/mL with the width of inhibition area being 24.67 ± 0.58^{d} (mm). Following by the extract at concentrations of 500 (20.33 ± 0.58 mm) and 250 mg/mL (18.00 ± 0.00 mm). Therefore, we concluded that the inhibition activity of the extract was concentration dependence. They are all significantly different from each other (P<0.05) by SNK test. Evidence from this study also revealed the stronger effect of the extract used at 1000 mg/mL than Gentamicin 10µg (23.00 ± 0.00) (p<0.05).

Table 2. The antibacterial activity of *T. tenuis* methanol extract on B. cereus

Extract (mg/mL)	Extract/paper disk (µL)	Extract amount/paper disk	Inhibition zone diameter(mm)
2% DMSO	20	0 mg	0.00 ± 0.00^{a}
250	20	5 mg	18.00 ± 0.00^{b}
500	20	10 mg	20.33 ± 0.58^{c}
1000	20	20 mg	24.67 ± 0.58^{d}
Gentamicin	-	10µg	23.00 ± 0.00^{e}

The different alphabet represents the significant difference between groups by One-way ANOVA test on rank followed by SNK test (P<0.05).

The antibacterial capacity of plants belonging to the genus *Tylophora* has been investigated so far. For instance, *T. indica* possesses a broad spectrum of activities against Gram-positive and Gram-negative bacteria. The crude ethanolic extract of *T. indica* leaves is able to depress the growth of bacteria and fungi including *K. pneumoniae, S. aureus, S. typhi, Proteus vulgaris, P. aeruginosa, E coli, A. niger, A. fumigatus,* and speices of *Fusarium* [15]. Aqueous extract of mixed leaves and stem of *T. indica* shows inhibition activity on *E. coli* and P. *aeruginosa*. When used along with tetracyllin, the extract improves antibacterial activity of antibiotics. The presence of active compound as steroids, cardiac glycosides, saponins, phenolics, and alkaloids may respond to the action [16] which are found in our study. However, *T. hirsute* extract possesses antiparasite and antifungal activities but shows no effect on *B. subtilis* or *Shigella flexenari* [17].

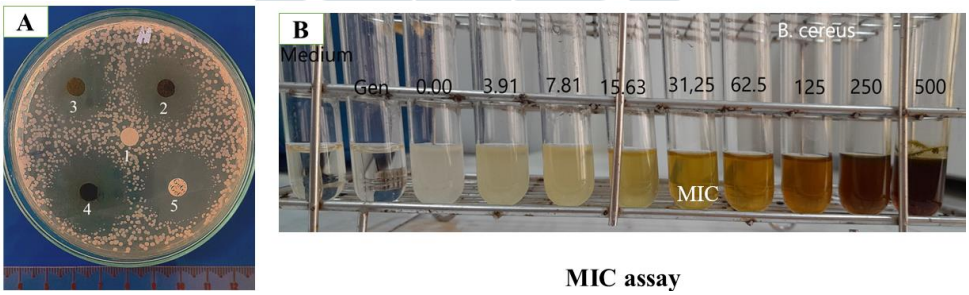


Figure 2. Agar diffusion test (A) and MIC assay of *T. tenuis* methanol extract on *B. cereus* (B)

Natural compounds identified from medicinal plants play a crucial role in biological activities, in particular, antibiotic activity. The underlying mechanisms of antibacterial activity of medicinal plant constituents are proven. Flavonoids have the ability to inhibit the synthesis of nucleic acids, inhibit the function of the cytoplasmic membrane, interfere membrane porins function, and killing of bacteria[18]. Saponins can bind to steroids on the surface of the endoplasmic reticulum resulting in the disruption of the biofilm system of the cell. Saponins cause changes in the structure of biological macromolecules in the cell [19]. Polyphenols are well-known for their antioxidant, antiinflammatory, and antimicrobial properties. Rutin, a member of polyphenols, can inhibit the formation of biofilm, down regulate *P. aeruginosa* virulence factors synthesis, and collapse cell membrane permeability[20]. Hence, the mechanism underlying antibacterial activity of *T. tenuis* can be vary due to multiple phytochemical compounds contain.

3.3 MIC, MBC and inhibition percentage of *T. tenuis* on bacteria growth

The minimum inhibition concentration and minimum bactericidal concentration of *T. tenuis* extract on *B. cereus* were measured using broth dilution method (**Fig. 2B**). MIC is defined as the lowest concentration of antimicrobial agents that will inhibit the visible growth of bacteria after overnight incubation and MBC is the lowest concentration of antibacterial agents required to

terminate bacterial survival [21]. In this study, the extract showed antibacterial activity in *B. cereus* with MIC value was 31.25 mg/mL and MBC value was 125.00 mg/mL. Although, these results are relatively high in the comparison with the MIC and MBC values reported by Jahan et al. while testing the antibacterial activity of T. indica (MIC ranging from 1.53 to 24.0 µg/ml with *E. coli, K. pneumoniae, P. aeruginosa, S. dysenteriae* type 1 and *S. typhi* [22]. Our values are comparable to the antibacterial activity of *Tanacetum vulgare* extract, which are from 53.9 to 125.9 mg/mL (tested strains *E. coli, S. agalactiae, T. vulgare, Streptococci*, and *Serratia liquefaciens* [23] or antibacterial activity of *Bixa orellana* stem, root, and leaves extract against *B. cereus* (MIC = 34.71; 28.18, and 14.11 mg/mL, respectively) [24].

IV. CONCLUSIONS

In conclusion, the present study indicates that methanolic extract of *T. tenuis* possessed antibibacterial effect on *B. cereus*, a Gram-positive bacteria, in vitro. The extract showed strong activity target to the growth of B. cereus at high concentrations which could be comparable to gentamicin. Bioactive compounds like polyphenols, alkaloids, flavonoids, saponins, and triterpenoids can provide antibacterial properties of the extract. This investigation suggests the prospect of the use of *T. tenuis* as a novel agent in infectious diseases control.

V. CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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