Evaluation of Antibacterial Activity of fractions from stem extract of *Tinospora crispa* (L.) Hook. f. & Thomson

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**Abstract.** The aim of this study is to evaluate the antibacterial activity of fractions from *Tinospora crispa* (L.) Hook. f. & Thomson stem extract. The extraction was fractionated by solvents with increasing polarity, then partitioned with acidic water and base to concentrate alkaloids. There are totally 7 fractions named PD-6, PD-K, PD-Cf2, PD-Cf1, PD-EtOAc, PD-BuOH and PD-N. With the help of disc diffusion method, 7 fractions were separately evaluated for their ability to inhibit the growth of some common bacterial species, including 3 Gram-positive and 3 Gram-negative bacteria. The result showed that *Tinospora crispa* was able to restrain the growth of the 3 Gram-positive species but not the 3 Gram-negative species. Antibacterial substances distributed in 6 out of 7 fractions tested, excluding PD-K fraction. PD-K sample mainly contained strong basic alkaloid and soluble water. Especially, weak basic alkaloids in PD-Cf2 were able to restrict the bacterial growth stronger than stronger basic alkaloids in PD-K. On the other hand, ethyl acetate fraction (PD-EtOAc), which contained medium polar compounds, were able to restrict the growth of bacteria slightly better than the others. It was subjected to traditional column chromatography to isolate some components. The purified substance was structurally identified by thin-layer chromatography, high performance liquid chromatography and spectroscopic data analyses.

1. **Introduction**

Antibiotic resistance is a global issue, especially in developing countries. However, current research to find new antibiotics and antifungal agents by chemical synthesis is unable to keep up with the diverse resistance of bacteria. To counteract the resistance of microorganisms, it is necessary to find new, more complex and often chemical structures widely distributed in many pharmaceuticals [1].

*Tinospora crispa* (L.) Hook. f. & Thomson (Menispermaceae) (*T. crispa*), also known as Ky Ninh cord, not only in Vietnam but also in India and used to treat a number of diseases such as malaria, cough, poor digestion, colitis, effective cure cancer and diabetes [2-3]. Traditionally, *T. crispa* is used to (1) treat jaundice, rheumatism, inflammation, fractures, scabies, hypertension (2) reduce feelings of thirst, (3) appetite and resistance and clear heat. Chemically, the Amulet contains alkaloids, flavonoids, and flavone glycosides, triterpene, diterpene and diterpene glycoside, cis-clerodan-furanoditerpenoid, lactone, sterol, lignan and nucleoside [4]. Berberin present in *T. crispa* had an anti-bacterial effect on *Helicobacter Pylori* and some other bacteria. In addition, numerous valuable compounds included, cinnamic acid, gallic acid, N-cisferuloyltyramine, and secoisolariciresinol, methyl 3,4-dihydroxybenzoate, apigenin, 3-O-β-D-glucopyranosyl-L-sitosterol have been isolated from the plant [5][6][7]. In parallel with the isolation studies of compounds in *T. crispa*, their antibacterial effects were also assessed. Mohammed et al. (2012) reported that ethanolic solvent extract had the largest antibacterial zone on two strains, *Streptococcus pneumonia* and *Escheria coli*, followed by extracts with CHCl₃, MeOH and water [8]. In addition, the report of L Yoga Latha (2006) showed that chloroform,
petroleum ether and methanolic extracts of the plant exhibited effective antibacterial properties against some Gram-positive and Gram-negative bacteria by using disc diffusion [9]. Although it has been evaluated as a source of medicinal materials with numerous biological and medicinal values, studies in terms of identifying specific chemical components with pharmacological effects, especially antibacterial properties, have remained lacking. Therefore, the aim of this study was to evaluate the antibacterial activity of different fractions from *T. crispa* crude stem extracts in order to enhance their potential use.

2. Materials and Methods.

2.1. Plant materials
The dried stalks of *T. crispa* were collected from Luan Duc shop, District 5, Ho Chi Minh City then ground into fine powder by high speed grinder machine (RRH-1000, Zhejiang, China). Figure 1 shows the biological stems of *T. crispa*.

![Biological stems of T. crispa](image)

**Figure 1.** Biological stems of *T. crispa*

2.2. Extraction and fractionation
The processes of extraction and fractionation of *T. crispa* stem extract were summarized in Figure 2 below. Briefly, dried and grounded aerial parts of *T. crispa* (4 kg) were extracted with 96% ethanol three times and macerated 24 h each time [2]. The solvent was allowed to evaporate using a rotary evaporator. The crude ethanolic extract was dissolved in water and acidified to pH 2 using H$_2$SO$_4$ 2%. The resulted solution was washed by n-Hexane, producing three layers of n-Hexane, acidic solution and the residue. The n-Hexane extract was evaporated under reduced pressure to produce n-hexane fraction (PD-Hex). The residue was dissolved in 20% MeOH and then successively partitioned by solvent-solvent fractionation into four major fractions which were chloroform (PD-Cf1), ethyl acetate (PD-EtOAc), n-butanol (PD-BuOH) and water (PD-N). The other three fractions from the acidic solution were basified to pH 10 using 10% NaOH and then extracted with chloroform in order to achieve chloroform fraction (PD-Cf2) and water fraction (PD-K) which supposedly contained alkaloids with strong alkalinity and soluble in water.

The partition process was carried out by continuous shaking in separating funnel until no residue was left after evaporation. In each fraction, the solution was evaporated under reduced pressure to eliminate solvents.

The PD-EtOAc extract was subjected to column chromatography (silica gel, 200–400 m mesh), eluted with gradients of chloroform–ethyl acetate and finally washed with chloroform to get pure substances.
Figure 2. Flow of liquid-liquid extraction procedure from T. crispa stem

2.3 Antimicrobial assay of T. crispa fractions
The present study evaluated the antibacterial activity of T. crispa fractions against Gram-positive (i.e. *Bacillus cereus* ATCC 25923, *Staphylococcus aureus* ATCC 13932) and Gram-negative bacteria (i.e. *Salmonella enterica* ATCC 14028, *Escherichia coli* ATCC 25922 and *Enterobacter aerogenes*) using the disc diffusion method as described in previous studies [10-11]. The bacterial strains were firstly grown and cultivated using Tryptone Soy Broth (TSB; Difco Laboratory Inc., Detroit, MI, USA) and corrected to a value of Mc Farland 0.5 equivalent to 1-2,108 with 0.9% NaCl physiological saline prior to the experiment. An aliquot of 100 µl of bacterial culture (10^8 CFU/mL) was spread evenly onto the surface of the plates containing Mueller – Hinton Agar environment – Merck (MHA; Difco Laboratory Inc., Detroit, MI, USA). Wells with a diameter of 6 mm was made and loaded with 50 µl of the sample extract (250 mg/ml). Dimethyl sulfoxide (DMSO) and tetracycline (0.25 mg/ml) were used as a negative and positive controls, respectively. After 16-18 h of incubation at 25°C, the antibacterial and antifungal activities were determined by measuring the diameter (mm) of growth inhibitory ring.

3. Results and discussion

3.1 Extraction and fractionation
Extraction of T. crispa stem (4 kg) involved three-time use of ethanol. The crude ethanolic extract resulted in the following fractions: PD-hex (17.98 g), PD-Cf1 (34.80 g), PD-EtOAc (5.35 g), PD-BuOH (9.66 g), PD-N (7.45 g), PD-Cf2 (11.98 g) and PD-K (350 g).

The antibacterial activity of fractions with an equal concentration (250 mg/mL) from T. crispa stem extracts on Gram-negative and Gram-positive bacteria were evaluated by measuring the inhibition zone diameter (mm). Overall, as shown in Table 1, all the fractions were unable to inhibit the growth of Gram-negative bacteria while exhibiting various inhibition against the Gram-positive ones. In particular, PD-Cf2 exhibited the highest inhibition against *B. cereus*, followed by PD-Hex, PD-Cf1, PD-EtOAc and PD-N and PD-BuOH extracts. These extracts also showed high inhibitory activity against *S. aureus* and *L. monocytogenes*, except for PD-N and PD-Hex whose activities were ineffective against *L. monocytogenes* and *S. aureus*. Extracts that are unable to inhibit the growth of these Gram-negative
strains may be due to differences in bacterial cell wall structure [12-13]. In Gram-positive bacteria, the cell wall is composed mainly of many peptidoglycan layers, yet in Gram-negative bacteria, beside peptidoglycan layer, their cell wall is also composed of and a lipopolysaccharide layer, which acts as a bacterial cell shield against harmful agents. This antibacterial result was similar to the report of A.I.C Mohammed and colleagues in 2012 that the CHCl₃ extract of Lanyard was unable to inhibit as the growth of Streptococcus pneumonia [8]. However, as can be seen in this study, the resistance was on both E. coli and S. enterica.

Table 1. Inhibitory activity against Gram-negative and Gram-positive bacteria of T. crispa fractions (250 mg/mL). The presented values are mean ± standard deviation (S.D.) of three measurements.

| Bacterial strains | Zone of Inhibition (mm) | PD- N | PD- EtOAc | PD- CF₁ | PD- CF₂ | PD- BuOH | PD- Hex | PD- K | Tetracycline | DMSO |
|-------------------|-------------------------|-------|-----------|---------|---------|----------|---------|-------|--------------|------|
| B. cereus 46      |                         | 13 ± 1.41 | 14.5 ± 0.70 | 15.5 ± 0.70 | 18 | 11.5 ± 0.70 | 16.5 ± 0.70 | NA | 19.75 ± 4.11 | NA |
| S. aureus ATCC 25923 |                       | 11.5 ± 0.70 | 12 ± 1.41 | 12 ± 1.41 | 14.5 ± 0.70 | NA | NA | NA | 28.25 ± 2.5 | NA |
| L. monocytogenes ATCC 13932 |                   | NA | 13.5 ± 0.70 | 16 ± 1.41 | 17 ± 2.82 | NA | 11 | NA | 34.5 ± 1.73 | NA |
| E. coli ATCC 25922 |                         | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| S. enterica ATCC 14028 |                       | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| E. aerogenes       |                         | NA | NA | NA | NA | NA | NA | NA | NA | NA |

NA: No Activity

Table 2. Antibacterial activity of T. crispa fractions
Comparing the diameter of bacterial growth inhibition zone with Table 2, the segments had moderate to strong antibacterial capacity (diameter > 10mm). Due to the less complex cell wall structure of Gram positive bacteria, PD-EtOAc, PD-CF1, PD-CF2 and PD-Hex fractions exhibited strong inhibition capacity against B. cereus 46 [14]. Similarly, PD-CF1 and PD-CF2 also inhibited L. monocytogenes growth [15]. The remaining segments were moderately inhibited on Gram-positive strains.

Among the strong antibacterial segments, the PD-CF2 segment contains alkaline alkaloid, because this fraction was extracted by the alkaloid extraction process, alkalized with Na₂CO₃ (the alkali agent of alkaline alkaloid) and then shaken with CHCl₃. This segment has a strong inhibitory ability on all 3 species of Gram positive bacteria.

Highly alkaline alkaloids were also concentrated in the P-K segment. This segment contained the
alkaloid after alkalization with Na$_2$CO$_3$ and converted into a water-soluble base (containing strong alkaline alkaloids, often protoberberin structure). However, the PD-K segment was unable to resist the tested Gram-negative and Gram-positive bacteria. This could be explained by the limited solubility in PD-K fraction of bioactive compounds, namely, tannins, polyphenols and flavonoids, which has led to low antimicrobial activity [16]. In conclusion, antibacterial substances were concentrated in solvents with polarity ranging from low to average such as n-Hexane, ethyl acetate, chloroform and n-butanol. This ability is relatively high compared the study of Muanza D. et al. (1994) [17].

4. Conclusion
In short, the present study investigated antibacterial effects of fractions of T. crispa stem extracts, which were produced by using weak to medium polar solvents such as n-Hexane, EtOAc and CHCl$_3$. All of these fractions were unable to counteract the growth of selected Gram-negative bacteria; while having varied inhibitory activity against the Gram-positive bacteria, depending on the solvent polarity. Future research is required to exploit the plant phytochemical profile as well as to study the underlying mechanism that have possibly been employed.

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