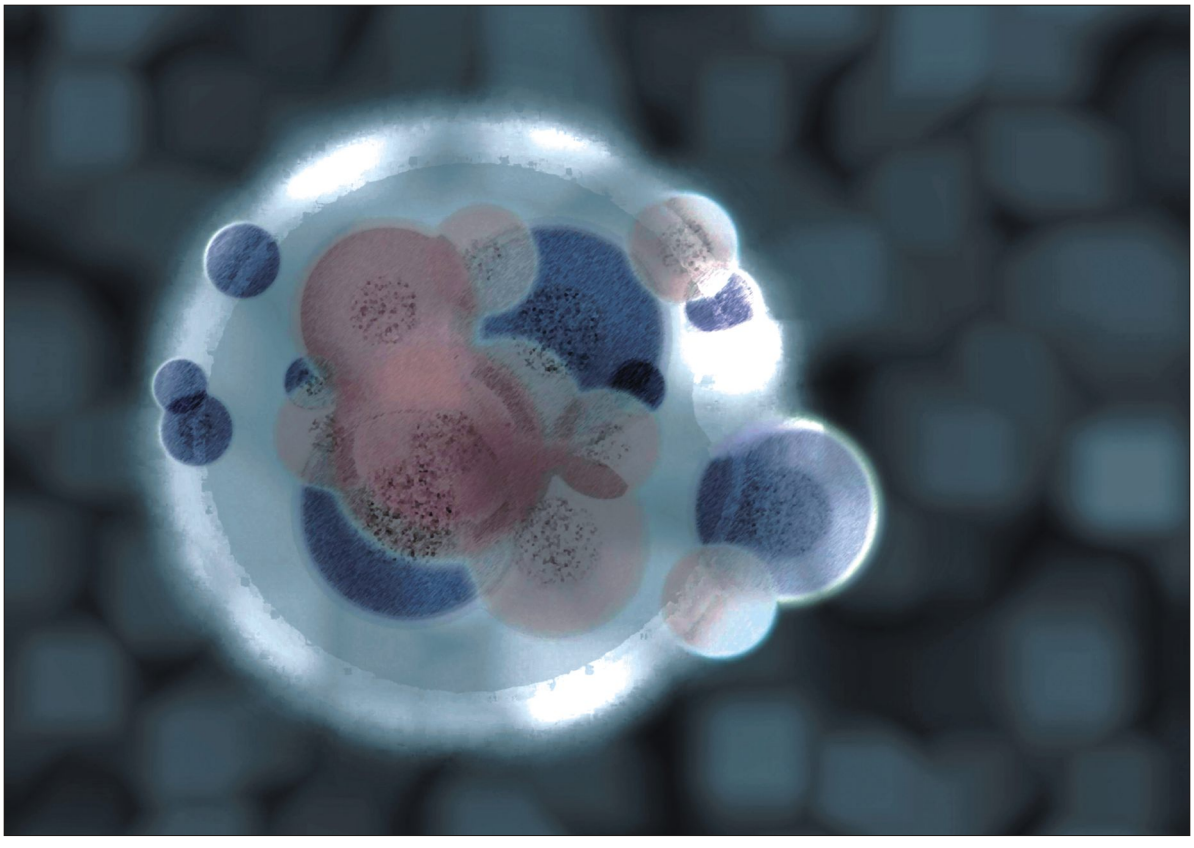

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Research Article
Evaluation of The Antimicrobial Activities of Crude Extracts of *Tinospora crispa* Stem

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Abstract

Background and Objective: Antimicrobial resistance has become a critical issue to healthcare institutions globally. The search for new and safer antimicrobial agents from natural resources has gained attention of many researchers nowadays. Antimicrobial activity of *Tinospora crispa* stem extracts on 6 bacterial species and 3 fungal species was evaluated. **Materials and Methods:** Bioactive compounds from *T. crispa* stem were extracted using 2 methods, maceration and sonication, and using 3 different solvents: chloroform, methanol and water. Antimicrobial activity was determined by disc diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). **Results:** Results showed that sonication yielded a slightly higher percentage of crude extracts for all solvents compared to maceration (The percentage of extract from maceration and sonication methods increased with increasing solvent polarity (chloroform<methanol<water). From the tests, the methanol extract had an MIC value of 100 mg mL^{-1} against *S. aureus* and *B. cereus* and an MBC value of 5.0 mg mL^{-1} against *S. aureus*. No activity was shown on the 3 tested fungal species. **Conclusion:** Hence it was concluded that crude extract of *T. crispa* stem showed a weak activity of inhibiting bacterial growth and did not inhibit the growth of fungus.

Key words: *Tinospora crispa* stem, antimicrobial activity, crude extracts, disc diffusion method, bacterial growth, inhibition zone

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Infectious diseases caused by pathogenic microorganisms are the main causes of death, affecting millions of people worldwide[1]. Infection by pathogenic microorganisms interferes with the immune system of human body, especially in immunocompromised patients. In the 20th century, Sir Alexander Fleming discovered penicillin, the most significant antibiotic that saved many lives during that time. However, the emergence of multidrug-resistant microorganisms has become a serious problem globally and has caused difficulties in treating infectious diseases[2]. This problem is caused by indiscriminate use of antibiotics[3] as well as the undesirable side effects of certain antibiotics[4]. This led the researchers into turning their interests to safer natural resources for development of new antimicrobial agents in combating pathogenic microorganisms.

For a long period of time, medicinal plants have been traditionally used to treat infectious diseases. According to the WHO[5], medicinal plants are the best source for new antimicrobial agents due to presence of bioactive compounds in the plants such as alkaloids and tannins[6,7], flavonoids, and phenolic compounds[6]. About 1,200-2,000 plant species in Peninsular Malaysia, Sabah, and Sarawak have medicinal properties that can be used to treat diseases caused by microorganisms[8]. Medicinal plants have been used over the years to treat various diseases such as diabetes, hypertension, cholera, rheumatism, and others. Bioactive components produced by most medicinal plants have been reported to show antimicrobial activity against many pathogenic bacteria[9].

Tinospora crispa is locally known as 'patawali' or 'akar seruntun' by the local people in Malaysia. It is a plant from the family of Menispermaceae. *Tinospora crispa* is also known as 'brotowali' or 'andawali' in Indonesia, 'makabuhay' in the Philippines, 'wan kab hoi yai' in Thailand, and 'kattukodi' in India[10,11]. This plant grows in the wild and is distributed in tropical and subtropical regions of Asia such as Indonesia, the Philippines, Thailand, India, and Vietnam[11]. *Tinospora crispa* has been widely used in Malaysia by the locals for the treatment of fever, jaundice, and diabetes[12]. Besides, it is also used for treatment of malaria and as a wound cleansing agent for rheumatic wounds and skin diseases[11]. Bioactive compounds such as resin, starch, glycoside, pikroretoside, paroxetine, berberine, alkaloids, and palmatine found in *T. crispa* act as a defence system against microorganisms[13]. Therefore, this study aimed at evaluating the antimicrobial activity of *T. crispa* stem extracts using different solvents (methanol, chloroform and distilled water) against selected microorganisms.

MATERIALS AND METHODS

Study area: The study was carried out during a period of 5 months, from February, 2019 until the end of June, 2019. The crude extracts of *T. crispa* stem was prepared in the laboratory of Environmental Chemistry, Department of Environmental Health and Industrial Safety, Universiti Kebangsaan Malaysia, Malaysia.

Preparation of plant: *Tinospora crispa* stem powder was obtained from Yuda Company Sdn Bhd at Rawang Selangor. The whole sample (1 kg) per packet was stored in a dry place at room temperature. Sixty gram of *T. crispa* stem powder was weighed using electronic weighting balance and used in this study.

Extraction: Three different types of solvents were used in two different extraction methods namely maceration and ultrasonication. The solvents used in this study consist of solvents from low polarity to high polarity (chloroform, methanol and aqueous).

Maceration: *Tinospora crispa* stems powder was extracted with 600 mL of chloroform, methanol and aqueous solution at a ratio of 1:10 and left to macerate for 3 days at room temperature with occasional shaking using orbital shaker. Then, extracts were filtered through Whatman paper No.1. The filtrates were concentrated under reduced pressure at 40-45°C using rotary evaporator and the dried crude extracts were weighed to calculate the yield and stored at (-20°C) until used for analysis.

Sonication: Extraction was performed using chloroform, methanol and aqueous solution by immersing the extracts into ultrasonic bath. Sonication was run for 30 min with ultrasound frequency >20 kHz. The filtrates were then concentrated using rotary evaporator to obtain the crude extracts. All collected extracts were stored at (-20°C) until use.

Preparation of sterile discs: Whatman filter paper No.1 was punched into 6mm discs and sterilized. Sterile discs were loaded with 10 µL of extracts (100 mg mL^{-1}) and left for hours in a sterile condition. The final concentration used for the tests was 1 mg/disc.

Test Microorganisms: Six species of bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa and Salmonela typhimurium*) and 3 species of fungi (*Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevisiae*) were tested in this study. Bacteria and fungi were obtained from Department of Biomedical Sciences , Faculty of Health Sciences Universiti Kebangsaan Malaysia (UKM) whilst *S. cerevisiae* was purchased from Faculty of Sciences and Technology, Universiti Kebangsaan Malaysia Bangi.

Microorganism culture: Each of the bacteria strains was cultured onto nutrient agar plate and incubated for 18-24 h at 37°C while fungi were cultured onto Sabouraud Dextrose agar plate for 2-3 days at 32°C. Two or three colonies were then cultured in 5 mL of Mueller Hinton Broth for 4 h. The optical density of bacteria culture was adjusted to 0.5 McFarland standard (1.0×10^8 CFU mL^{-1}) while for fungus was 1.0×10^6 CFU mL^{-1} using spectrophotometer measurements.

Determination of antimicrobial activity: Antimicrobial activity was determined by disc diffusion method. Bacterial and fungal culture adjusted to 0.5 McFarland standard was used to lawn Mueller- Hinton and Sabouraud Dextrose agar plates using sterile swabs respectively. These plates were then left to dry for 2-3 min before sensitivity test. The discs which were impregnated with extracts were placed on the Mueller-Hinton and Sabouraud Dextrose agar. Each of the test plate consisted of 5 discs, including 1 positive control (Gentamicin and Vancomycin) for bacteria while Nystatin for fungus, 1 negative control (DMSO 5%) and 3 extract-treated discs. The plate was incubated for 24 h at 37°C for bacteria and 2-3 days at 32°C for fungus. Then, the diameter of inhibition zone was measured by using ruler. The test was done in triplicates.

Minimum inhibitory concentration (MIC): Minimum inhibitory concentration (MIC) was determined using 96-well microtiter plates. Briefly, 50 µL of the methanolic extracts was mixed with 50 µL of Mueller-Hinton Broth before adding the standard inoculated bacterial culture into the mixture. The lowest extract concentration that did not show any bacterial growth for *B. cereus* and *S. aureus* was considered as MIC value.

Minimum bactericidal concentration (MBC): The test mixture concentration which did not show any growth of bacteria in *S. aureus* and *B. cereus* obtained from determination of MIC were subcultured onto Mueller Hinton Agar and incubated for 24 h at 37°C. The least concentration with no growth of these bacteria was considered as the MBC value.

RESULTS

The percentage yield of *T. crispa* stem extract from maceration using aqueous solution was the highest (11.0%), followed by methanol (3.0%) and chloroform (2.02%) respectively as shown in Table 1. Meanwhile, sonication method produced the highest percentage of extracts from aqueous extraction (11.78%), followed by methanol extraction with 3.15% and chloroform extraction with 2.68%. Sonication yielded a slightly higher percentage of extract as compared to maceration for all the solvents used as shown in Table 2.

From the antimicrobial tests, it was observed that methanol extracts obtained from maceration alone showed antibacterial activity against 2 g positive bacteria (*B. cereus* and *S. aureus*) but did not show any activity against *B. subtilis*. Diameter of inhibition zone for the methanol extract were 4.0±0.5 mm for *B. cereus* and 8.3±0.6 mm for *S. aureus* at the concentration of 100 mg mL^{-1} (1.0 mg/disc). However, chloroform and aqueous extracts did not show any antibacterial activities against Gram positive bacteria. Furthermore, all *T. crispa* extracts showed no inhibition against Gram negative bacteria (*E. coli*, *P. aeruginosa* and *S. typhimurium*). Extracts from sonication using all extraction solvents exhibited no inhibition against all tested microorganisms. Moreover, *T. crispa* stem extracts from both maceration and sonication also failed to exhibit any antifungal activity against the tested fungal species as shown in Table 3.

Minimum inhibition concentration (MIC) test was performed for methanol extract against selected bacteria i.e., *B. cereus* and *S. aureus*. The MIC value for *B. cereus* and *S. aureus* using standard antibiotics was 1.0 mg mL^{-1} and using methanol extract was 100 mg mL^{-1} . Methanol extract showed MBC value at 5.0 mg mL^{-1} against *S. aureus* alone.

Table 1: Percentage of *T. crispa* crude extract using maceration method

Solvents	Weight of extract (g)	Yield of crude extract (%)
Chloroform	1.21	2.02
Methanol	1.80	3.00
Aqueous	6.60	11.00

Table 2: Percentage of *T. crispa* crude extract using sonication method

Solvents	Weight of extract (g)	Yield of crude extract (%)
Chloroform	1.61	2.68
Methanol	1.89	3.15
Aqueous	7.07	11.78

Table 3: Diameter of inhibition zone by *T. crispa* stem extract prepared using maceration method

	Inhibition zone diameter (mm)			
		Extract		
Bacteria	Standard antibiotic (1 mg mL^{-1})	Chloroform	Methanol (100 mg mL^{-1})	Aqueous
Gram positive				
B. cereus	24.7±0.6	0.0±0.0	4.0±0.5	0.0±0.0
B. subtilis	24.7±0.6	0.0±0.0	0.0±0.0	0.0±0.0
S. aureus	19.7±0.6	0.0±0.0	8.3±0.6	0.0±0.0
Gram negative				
E. coli	24.7 ±0.9	0.0±0.0	0.0±0.0	0.0±0.0
S. typhimurium	23.0±0.8	0.0±0.0	0.0±0.0	0.0±0.0
P. aeruginosa	24.3±0.6	0.0±0.0	0.0±0.0	0.0±0.0

DISCUSSION

The basis for choices of solvents used in this study is based on their polarity. The polarity index of the each selected solvent is shown in Table 2. Extraction of *T. crispa* stem resulted in highest percentage yield of crude extracts using aqueous solvent compared to methanol and chloroform for both maceration and sonication methods. Aqueous solvent is effective in extracting dissolved chemical compounds because water has a high polarity and shorter chain which helps in increasing the ability to extract polar compounds[14]. The percentage of aqueous extracts for the sonication method (11.78%) was slightly higher compared to the maceration method (11.0%). The sound waves generated during the sonication process can help cavitation that will increase the mass transfer of the cell contents[15].

From this study, methanol extract from maceration method showed weak inhibitory activity against *S. aureus* and *B. cereus* with average diameter of the inhibition zone $\leq$9 mm. The results from this study were similar to the findings by Islam *et al.*[16]. The study showed a little inhibition zone of methanol extract at the concentration of 0.4 mg/disc against *S. aureus* (7.9 mm), *B. cereus* (4.6 mm), *E. coli* (2.8 mm) and *P. aeruginosa* (3.5 mm). Another study showed no inhibition zone against selected microorganisms (*B. cereus, E. coli, P. aeruginosa and Candida albicans)* at the concentration[17] 0.5, 1.0, 2.5, and 5.0 mg disc^{-1}.

There are several factors that could have contributed to the negative results seen for disc diffusion study here. Bioactive compounds in the crude extracts can only show antibacterial activity at sufficient amount of concentration[18]. In addition, Sulaiman *et al.*[17]stated that there is a probability that the bioactive compounds extracted through maceration are insufficient to inhibit the growth of tested microorganisms.

Sonication method showed no antibacterial activity against all the bacterial cultures. The bioactive compounds in crude extract of *T. crispa* are inactivated by sound waves generated during sonication process[19]. This is due to the formation of free radicals that can damage the molecular structure of bioactive compounds[20].

Meanwhile, chloroform and aqueous extracts did not show any antibacterial activity against the bacteria tested for both extraction methods. The percentage yield of aqueous extract was higher than methanol and chloroform extraction but only methanol extracts showed antibacterial activity in this study. This shows that antibacterial activity is unaffected by the higher yield of the crude extracts but depends on the polarity of solvent which can influence the bioactive compound extracted from the *T. crispa* stem[21]. Methanol extract showed antibacterial activity against *B. cereus* and *S. aureus* alone suggesting that bioactive compounds in *T. crispa* stems can be extracted through methanol alone[22]. Methanol may dissolve polar compounds such as flavonoids, polyphenols, tannins and saponins[23]. Tannins extracted using methanol had shown antimicrobial activity. Tannin can damage the polypeptide compound on the cell wall of bacteria and interfere with the transmission of protein sources to inhibit bacterial growth[24].

Inhibition showed in this study was more sensitive to Gram positive bacteria compared to Gram negative bacteria. The results of this study were similar with study conducted by Cowan[23], who reported that Gram negative bacteria are more resistant. According to Von Staszewski *et al.*[25], bacterial cell wall of Gram negative is more complex comprising of phospholipid membranes and peptidoglycan layers that prevent antimicrobial substances from penetrating them. Nevertheless, no antibacterial activity was demonstrated by all the crude extracts against *B. subtilis* (Gram positive). The results of this study were supported by Gami and Parabia[26] who reported that methanol extract of *Tinosporacordifolia* stem is inactive against *B. subtilis*. These bacteria are less sensitive due to their ability to form endospores that make them resistant to the environment[27].

No antifungal activity was shown for both extraction method using all extracts against selected fungi. The resistance of the fungus to the extract was likely due to the differences in mechanisms between fungi and bacteria. According to Ghannoum and Rice[28], fungus has a distinct diploid structure consisting of ergo sterol, the most important component of the cell membrane that prevents antifungal materials from penetrating the fungal cells.

CONCLUSION

The percentage of yield for aqueous extract of *T. crispa* using maceration and sonication methods was higher compared to that from chloroform and methanol extraction. Sonication method was a better extraction method whereby the percentage of yield obtained for all the solvents was slightly higher than for the maceration method. The methanol extract obtained using maceration alone showed low antibacterial activity against *S. aureus* and *B. cereus* with inhibition zones of 4.0±3.5 and 8.3±0.6 mm respectively, at the concentration of 100 mg mL^{-1} (1.0 mg/disc). The methanol extract had an MIC value of 100 mg mL^{-1} against *S. aureus* and *B. cereus* and an MBC value of 5.0 mg mL^{-1} against *S. aureus*. The methanol extract acted as a bacteriostatic agent against *B. cereus* and as bactericidal agent for *S. aureus*.

SIGNIFICANCE STATEMENT

This study discovered the active compounds in the crude extracts of *T. crispa* can be beneficial for human body to act as anti-therapeutic agent and antimicrobial agents. Various studies show that *T. crispa* extracts contain many phytochemicals that can be used to treat various kind of diseases. This study will help the researchers to uncover the critical areas of other parts of *T. crispa* that many researchers were not able to explore. Thus, the new and safer antimicrobial agents from medicinal plants can be developed.

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