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Antibacterial activity of plant extracts in different solvents against pathogenic bacteria: An *in vitro* experiment

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ABSTRACT

Objective: To assess the antibacterial activity of 5 selected plants against 4 pathogenic bacteria.

Methods: Three solvents with different polarities were used to extract antimicrobial agents from the plants *via* maceration technique. The agar-disc diffusion technique was adopted to primarily screen antibacterial activities. Broth-dilution assay was employed to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

Results: Among all extracts, the ethanol extract of *Piper betle* Linn showed the highest antibacterial activity against Gram-positive and the negative bacteria. MIC and MBC of the ethanol extract of *Piper betle* Linn against *Salmonella typhimurium* were the same (1 562.50 mg/L); while it showed the highest MIC and MBC against *Pseudomonas aeruginosa* of 6 250 mg/L and 12 500 mg/L, respectively.

Conclusions: *Salmonella typhimurium* is the most susceptible bacteria while *Pseudomonas aeruginosa* is the most resistant bacteria towards the ethanol extract of *Piper betle* Linn. *Piper betle* possesses compounds with potential antibacterial activity and might be useful as an alternative to control infectious diseases.

KEYWORDS: Antibacterial activity; Plant; Organic solvent; Pathogenic bacteria

1. Introduction

Infectious diseases have been recognized as one of the major intimidations to human health throughout the world. Most of them are caused by microorganisms such as bacteria, viruses, rickettsia, and fungi[1]. It is reported that bacteria are attributed to approximately up to 30% of all diseases, leading to millions of deaths every year[2]. To respond to the threat from microbial diseases, synthetic antibiotics have been extensively developed and introduced to pharmaceutical markets. However, overuse and misuse of synthetic antibiotics have gradually resulted in drug-resistant bacteria, consequently raising a newly global therapeutic challenge in the public health system, called antibiotic resistance[3].

To overcome the problem of bacterial infection, effective and safe antibacterial agents must be identified and pursued. Active compounds with antibacterial activity have been identified from plants so far to develop new promising drugs. Compared with synthetic drugs, plant-based antibiotics are considered to be safer due to their natural origin. In addition, plants are rich in numerous secondary metabolites such as tannins, lignin, carotenoids, flavonoids, and alkaloids, which are relatively smaller in quantity comparing with primary metabolites such as carbohydrate, protein, and lipid. Although these compounds are non-nutritive agents, they are believed to have antimicrobial function[4-6]. In Thailand, many plants have been widely used as remedies for ages. Such plants are easily available, inexpensive, and safe, making them increasingly popular and suitable for pharmaceutical purposes[7]. Therefore, this study aimed to screen the antibacterial activity of some selected Thai medicinal plants against *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), and *Salmonella typhimurium* (*S. typhimurium*).

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2. Materials and methods

2.1. Extraction

According to traditional Thai folk medicines, different parts of 5 selected plants were used in this study. Leaves of *Cissampelos pareira L. var. hirsuta* (Buch. ex DC) Forman, *Stemona tuberosa* Lour, *Barringtonia acutangula* Gaertn, and *Piper betle* Linn as well as heartwoods of *Artocarpus lacucha* Buch-Ham were chosen for extraction. Plant materials were washed with tap water and subsequently dried at 60℃ by hot air oven. Afterward, they were grounded into powder and sieved through nylon membrane. The homogeneous powder was used for solvent extraction.

All plant samples were extracted with three kinds of solvents with different polarities, including water, ethanol, and hexane through maceration technique. In brief, each plant sample was mixed with solvent in a ratio of 1:4, and then the mixture was put into the orbital shaker. It was shaken at 150 rpm for 24 h for extraction. Residues were removed from the supernatant by using the No.1 Whatman filter paper. The supernatant was subsequently removed from the solvent *via* rotary evaporator. Dried crude extracts were finally obtained and kept in dark at 4℃ before use.

2.2. Antibacterial assay

The agar-disc diffusion method was employed for antibacterial activity screening. The bacteria strains used in this study were *S. aureus* TISTR 746 (referred as *S. aureus*), *E. coli* TISTR 117 (referred as *E. coli*), *P. aeruginosa* TISTR 1287 (referred as *P. aeruginosa*) and *S. typhimurium* TISTR 1469 (referred as *S. typhimurium*). These strains were obtained from the division of Thailand Institute of Scientific and Technological Research, Thailand, and grew in nutrient broth at 37℃ for 16-18 h to obtain bacterial concentration of 1×10^8 CFU/mL. Sterile blank discs with 6 mm diameter were individually placed on nutrient agar plate covered with 100 μL of the bacteria strain. Ten microliters of crude extract at 500 g/L in dimethyl sulfoxide were put into the sterile blank disc. These plates were incubated at 37℃ for 24 h. The antimicrobial activity was determined in triplicate by measuring diameter of inhibition zone (mm). Oxytetracycline (30 μg/disc) was used as the positive control. Water and dimethyl sulfoxide were used as negative control.

2.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Based on the results of the antibacterial testing , the most efficient crude extract was chosen to identify its MIC. Serial dilution was adopted to prepare various concentrations of the selected crude extract. The serial dilution technique was used to prepare various concentrations of *Piper betle* Linn extracts. The first concentration was 25 000 mg/L. Serial dilution was done for 8 times, and the range of concentration was 195-25 000 mg/L. After incubation for 16-18 h at 37℃. the growth of bacteria were checked in order to determine the MIC and MBC. The MIC value is defined as the lowest concentration of the crude extract with no visible growth of bacteria strain. To determine the MBC value, 100 μL of the mixture was plated onto the nutrient agar. The lowest concentration of crude extract with no growth of bacteria strain on the agar plate is defined as MBC value.

2.4. Statistical analysis

Statistix version 8.0 was used for statistical analysis. Data were expressed as mean ± standard deviation. Measurement data with normal distribution were analyzed using the one-way analysis of variance (ANOVA). The significant level of the test was set at $\alpha=0.05$.

3. Results

3.1. Antibacterial activity

The extracts from *Cissampelos pareira L. var. hirsuta* (Buch. ex DC) Forman showed no antibacterial activity against any bacteria strain. The remaining extracts prepared in solvents other than ethanol either gave no activity or lower activity than ethanol. The highest inhibition zones were found in the ethanol extract of *Piper betle* Linn. Its diameters of inhibition zones against *S. aureus*, *E. coli*, *P. aeruginosa* and *S. typhimurium* were (21.02±0.73) mm, (25.76±0.64) mm, (13.74±0.21) mm and (27.10±0.57) mm, respectively (Table 1).

No inhibitory effect was found in the extracts of *Stemona tuberosa* Lour and *Cissampelos pareira L. var. hirsuta* (Buch. ex DC) Forman gainst *S. aureus* (Gram-positive bacteria). Variable degrees of antibacterial activity were found from the ethanol extracts of *Artocarpus lacucha* Buch-Ham, *Barringtonia acutangula* Gaertn, and *Piper betle* Linn, with inhibition zone diameters as (15.44±0.42) mm, (9.51±0.53) mm and (21.02±0.73) mm, respectively. The ethanol extract of *Piper betle* Linn showed the highest anti-*S. aureus* activity among all extracts.

The ethanol extract of *Piper betle* Linn showed the highest anti-*E. coli*, anti-*P. aeruginosa* and anti-*S. typhimurium* activity among plant extracts, and its anti-*E. coli* and anti-*S. typhimurium* activities were significantly better than the antimicrobial efficiency of positive control oxytetracycline ($P<0.05$). Although the inhibition zone diameters of ethanol extract of *Piper betle* Linn. against *P. aeruginosa* was not greater than the positive control, it was still significantly higher than *Barringtonia acutangula* Gaertn. Notably, anti-*E. coli*, anti-*P. aeruginosa*, anti-*S. typhimurium* and anti-*S. aureus* activity varied among different solvents.

Table 1. Inhibition zone diameters of crude extracts.

Crude extract	Solvent	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhimurium*
Stemona tuberosa Lour	Hexane	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Stemona tuberosa Lour	Ethanol	6.00±0.00^{e}	11.61±0.80^{d}	6.00±0.00^{e}	6.00±0.00^{e}
Stemona tuberosa Lour	Water	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Artocarpus lacucha Buch.-Ham	Hexane	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Artocarpus lacucha Buch.-Ham	Ethanol	15.44±0.42^{c}	16.10±0.50^{c}	6.00±0.00^{e}	6.00±0.00^{e}
Artocarpus lacucha Buch.-Ham	Water	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Cissampelos pareira L. var. hirsuta (Buch. ex DC) Forman	Hexane	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Cissampelos pareira L. var. hirsuta (Buch. ex DC) Forman	Ethanol	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Cissampelos pareira L. var. hirsuta (Buch. ex DC) Forman	Water	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Barringtonia acutangula Gaertn	Hexane	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Barringtonia acutangula Gaertn	Ethanol	9.51±0.53^{d}	6.00±0.00^{e}	10.66±0.28^{c}	10.64±0.75^{d}
Barringtonia acutangula Gaertn	Water	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Piper betle Linn	Hexane	9.62±0.12^{d}	11.06±0.32^{d}	6.00±0.00^{e}	18.73±0.96^{c}
Piper betle Linn	Ethanol	21.02±0.73^{b}	25.76±0.64^{a}	13.74±0.21^{b}	27.10±0.57^{a}
Piper betle Linn	Water	8.70±0.31^{d}	11.75±0.79^{d}	8.05±0.36^{d}	9.51±0.37^{d}
Oxytetracycline (30 μg/disc)		23.44±0.78^{a}	24.22±0.90^{b}	20.69±0.78^{a}	23.98±0.47^{b}
Water		6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}
Dimethyl sulfoxide		6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}	6.00±0.00^{e}

Note: Results expressed as mean ± SD (mm). a-e: Mean values in the same column with different letters differ significantly (P<0.05). *S. aureus*: *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. coli*: *Escherichia coli*; *S. typhimurium*: *Salmonella typhimurium*.

3.2. MIC and MBC

Since that ethanol extract of *Piper betle* Linn showed highest antibacterial activity, the ethanol extract of *Piper betle* Linn was chosen for MIC and MBC determination.

The MIC of ethanol extract of *Piper betle* Linn against *S. aureus*, *E. coli*, and *S. typhimurium* were all 1 562.50 mg/L, while the MIC against *P. aeruginosa* was 6 250 mg/L. The MBC of ethanol extract of *Piper betle* Linn against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhimurium* were 6 250 mg/L, 3 125 mg/L, 12 500 mg/L, and 1 562.50 mg/L respectively.

4. Discussion

Our study shows that ethanol is the most suitable solvent for extraction because of its solvent polarity[8]. Ethanol and water contain hydroxyl group, which is able to form a hydrogen bond with the phytochemical compounds; while hexane belongs to hydrocarbon group and it is hard to form a hydrogen bond. Different phytochemical compounds are likely to dissolve into different solvents due to their natural properties[9].

The ethanol extract of *Piper betle* Linn showed the highest antibacterial activity against all bacteria strains. The inhibition zone diameters decreased 2-3 times when using water as a solvent. It indicated that antibacterial activity could not be improved when the polarity is increased[10].

The antibacterial activities of the ethanol extract of *Piper betle* Linn might be attributed to various photochemical agents such as steroids, diterpenes, tannin, flavonoids, saponin, coumarin phenolic compounds, volatile oils, fatty acids, and hydroxyl fatty acids[11-13]. These compounds have shown antimicrobial activities against bacteria and fungi *via* copious mechanisms, *e.g.* interrupting microbial membranes, weakening cellular mechanisms, controlling biofilm formation, inhibiting bacterial capsule production, and reducing microbial toxin production[8,12-18]. According to Burfield, essential oil from *Piper betle* leaves was dominated by phenylpropanoids and aromatic compounds such as eugenol, carvacrol, and chavicol, which account for antibacterial, antifungal, antiseptic effect. In our study, ethanol extract of *Piper betle* Linn might possess similar compounds, and the antibacterial activity might be ascribed to synergistic interaction among different compounds. In Thai folk medicine, *Piper betle* leaves have been traditionally used to treat oral malodor, pulmonary afflictions, and bleeding[19-21].

Our study suggested that *S. typhimurium* was the most sensitive bacteria to ethanol extract of *Piper betle* Linn while the most resistant bacterial was *P. aeruginosa*. The susceptibility of microorganisms toward *Piper betle* Linn extract was *S. typhimurium* > *E. coli* > *S. aureus* > *P. aeruginosa*. It implies that the crude ethanol extract of *Piper betle* Linn has a broad range of antibacterial activities. However, other studies show different MIC and MBC of this extract[14,16-18]. The possible reason is that the phytochemical components from plants vary from geological areas, harvesting seasons, climate, period of plant collection, parts of plant (*e.g.* leaves, flowers, stem, heartwoods), and extraction method. Besides, different microorganisms may also affect results[22-24].

The ethanol extract of *Piper betle* Linn exhibited antibacterial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhimurium*, and could be developed as an alternative antibacterial drug.

Conflict of interest statement

The authors report no conflict of interest.

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Authors' contributions

K.R. design the experiment; N.S. data collection; N.S. and K.R. data analysis; N.S and K.R. drafting the manuscript; K.R. final approval of the article.

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