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# Antimicrobial Activity of Medicinal Plant Extracts against Foodborne Spoilage and Pathogenic Microorganisms

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## ABSTRACT

Crude ethanol extracts from dried *Phyllanthus niruri* (DPN), fresh (FPB) and dried *Piper betle* Linn. (DPB) were tested for their inhibitory activity against three foodborne pathogenic microorganisms (*Escherichia coli*, *Staphylococcus aureus* and *Salmonella derby*) and five foodborne spoilage microorganisms (*Pseudomonas aeruginosa*, *Bacillus subtilis*, *Lactobacillus sp*., *Saccharomyces cerevisiae* and *Aspergillus niger*). Screening for antimicrobial activity using disc diffusion assay showed the inhibition of all tested microorganisms by DPB. On the other hand, only two of the test microorganisms (*S. aureus* and *Lactobacillus sp*.) were inhibited by FPB. Likewise, *Lactobacillus sp.* was the only test microorganism inhibited by DPN. Minimum inhibitory concentrations (MIC's) of the extracts were determined using agar dilution method on the same test microorganisms. Extract from DPB gave MIC values ranging from 160 to 10,240 parts per million (ppm). Results showed *S. cerevisiae* as the most sensitive and *B. subtilis* as the least sensitive to the extract. Both FPB and DPN extracts did not show growth inhibition of test microorganisms at the highest concentration used (10,240 ppm).


**Key words:** *Piper betle* Linn., *Phyllanthus niruri*, antimicrobial substance, minimum inhibitory concentration (MIC)

## INTRODUCTION

Food infection and intoxication are considered as the most common causes of foodborne diseases worldwide. Foodborne pathogens causing these diseases find their way in foods through cross contamination, improper handling and temperature abuse. *Staphylococcus aureus*, *Salmonella sp*. and *Escherichia coli* are among the common foodborne microorganisms that cause infection and intoxication. Food spoilage microorganisms, on the other hand, cause products to lose their quality which renders them unacceptable to consumers. Short shelf-life of food products because of spoilage is one of the major problems of the food industry. Examples of food spoilage microorganisms include *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Lactobacillus sp*., *Saccharomyces cerevisiae* and *Aspergillus niger* (Jay, 2000; Ray, 2001).

Prevention of pathogenic and spoilage microorganisms in foods is usually achieved by using chemical preservatives. These chemical preservatives act as antimicrobial compounds which inhibit the growth of undesirable microorganisms. However, the onset of increased

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demand for minimally-processed, extended shelf-life foods and reports of chemical preservatives as having potential toxicity demand food manufacturers to find alternative sources of antimicrobial compounds (Conner, 1993; Nychas, 1995).

In 1998, the World Health Organization estimated that 80% of the people living in developing countries almost exclusively use traditional medicine. Most of traditional medicine relies heavily on medicinal plants (Eloff, 1998). Two of the commonly used medicinal plants in tropical Asia are *Piper betle* Linn. and *Phyllanthus niruri.*

*Piper betle* Linn. belongs to the family Piperaceae. Its common names are betel vine, *phlu* and *see-keh*. Ethnomedicinal uses of *Piper betle* Linn. parts include treatment of disorders of physiological function, endo-parasites, skin diseases, eye diseases, urticaria, bronchial sputum occlusion and promotion of healthy teeth and skin (Farnsworth and Bunyapraphatsara, 1992).

*Phyllanthus niruri* belongs to the family Euphorbiaceae. It is known around the world as a natural remedy. Standard infusion or weak decoction of the plant or its aerial parts is used to treat types of biliary and urinary conditions such as kidney and gallbladder stones, hepatitis, cold, flu, tuberculosis and other viral infections. It is also used to treat other liver diseases and disorders, bacterial infections, diabetes and hypertension. Clinically, it has been proven that *Phyllanthus niruri* is an antihepatotoxic, antilithic, analgesic, hypotensive, antispasmodic, antiviral, antibacterial, diuretic, antimutagenic and hypoglycemic (Taylor, 2003).

Researches on antibacterial and antimycotic effects of spices and herbs used as seasoning agents in foods and beverages have been done extensively. Garlic, onion, leek, cinnamon, allspice, clove, oregano, thyme, savory, celery, parsley and angelica were some of the subjects of these researches (Beuchat and Golden, 1989; Conner, 1993; Elgayyar *et al*., 2001). In addition, basil is being studied as a source of antimicrobial agents that could be used in antimicrobial packaging for food preservation (Suppakul *et al.*, 2003). Extracts of 26 species of edible plants from China, Japan, Thailand and Yemen were also screened by Alzoreky and Nakahara (2002) for their antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella infantis*. However, only a few studies focused on the potential of medicinal plants as sources of antimicrobial compounds that could inhibit foodborne spoilage and pathogenic microorganisms. It was the aim of this study to determine the antimicrobial activity of extracts from medicinal plants against foodborne spoilage and pathogenic microorganisms.

## MATERIALS AND METHODS

### Preparation of freeze-dried extracts

Leaves of *Piper betle* Linn. plant (Figure 1) were purchased from Samyak Kaset, a local market along Ngamwonwan Road, Chatuchak, Bangkok. The leaves were thoroughly washed in running water prior to cutting into small pieces (ca $1 \times 1$ cm). The cut leaves were divided into two lots:

**Figure 1** Leaves of *Piper betle* Linn. plant.

fresh *Piper betle* Linn. (FPB) and dried *Piper betle* Linn. (DPB). Leaves for FPB lot was weighed into 20-g samples and placed in a glass container with cover. Extraction was done by adding 100 ml of 95% ethanol in each sample and then placing the sample in a water bath maintained at 60°C for 48 h (Jaturapronchai, 2003).

Prior to extraction, the cut leaves for the DPB lot was evenly distributed in aluminum trays and placed inside an incubator at 50 ± 2°C for 36 h. The dried leaves were then ground using an osteorizer. Ground dried leaves were weighed into 20-g samples and extracted by using the procedure outlined above.

Crude ethanol extracts from FPB and DPB lots were filtered through a Whatman No. 4 filter paper and distributed into dark bottles. The solvent was removed from the sample by using a rotary vacuum evaporator (Eyela, Tokyo Rikakikai Co., Ltd., Japan). The sample was rotary evaporated at 50°C until it reached $^1/_4$ its volume. Eighty ml of distilled water was then added to the sample and continuously rotary evaporated until it reached 50-ml volume (Jaturapronchai, 2003).

Ten percent (w/v) maltodextrin was added to the sample after rotary evaporation. Then, the sample was dried overnight (ca 20-24 h) in a freeze drier (Freezemobile, Model 5SL, The Virtis Company, Inc., New York, USA) (Jaturapronchai, 2003). After freeze-drying, the sample was placed in tightly sealed dark bottle and stored in desiccator until use.

*Phyllanthus niruri* plants (Figure 2) were collected from the field around Bangkok. Prior to drying, the plants were thoroughly cleaned in running water. Whole-plant materials were dried in incubator at 50 ± 2°C for 36 h and were grinded using a hammer mill. The procedure used in extraction of the dried *Phyllanthus niruri* (DPN) was the same with FPB and DPB but at room temperature (ca 26 ± 2°C) for 24 h. Rotary evaporation and freeze-drying procedures and storage conditions were the same as described above except for the amount of maltodextrin added to the sample prior to freeze-drying which was 5% (w/v).

**Preparation of test microorganisms**

The test microorganisms used were *Escherichia coli*, *Staphylococcus aureus*, *Salmonella derby*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Lactobacillus sp.*, *Saccharomyces cerevisiae* and *Aspergillus niger*. All of the test microorganisms were obtained from the Department of Microbiology, Faculty of Science, Kasetsart University.

Loopful of test microorganism was transferred to 10-ml Nutrient Broth (NB) and incubated for 24 h at 37 ± 1°C for bacteria or 30 ± 1°C for yeast. Optical density of the 24-h culture was measured at 625 nm ($OD_{625}$) using a spectrophotometer (BioMate 3, Thermo Spectronic, New York, USA). $OD_{625}$ was then adjusted to 0.1 by adding NB (Alzoreky and Nakahara, 2002). The prepared culture was appropriately diluted to achieve an inoculum size of approximately $10^6$ cfu/ml. Appropriate dilution was determined after spread plating the adjusted culture on Plate Count Agar (PCA) for bacteria or acidified Potato Dextrose Agar (PDA) for yeast

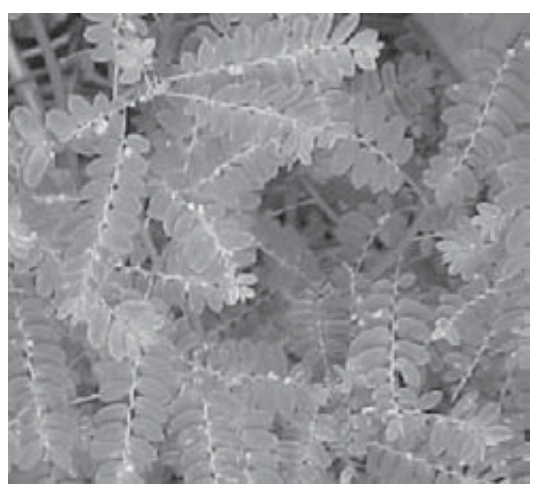

**Figure 2** *Phyllanthus niruri* plant.

using standard methods (Swanson *et al.*, 1992; Mislivec *et al.*, 1992).

*A. niger* was prepared as follows. Loopful of the mold was subcultured on acidified PDA slants at 30 ± 1°C for 5 d. The slants were then washed with 10 ml 0.05% Tween 80 to obtain a spore suspension (Bullerman *et al.*, 1977). $OD_{625}$ of the spore suspension was measured and adjusted to 0.1 by using 0.05% Tween 80 to achieve an inoculum size of $10^5$ cfu/ml. Inoculum size was confirmed by spread plating on acidified PDA using standard methods (Mislivec *et al.*, 1992).

All the culture media (broth and agar) and Tween 80 were purchased from Scharlau Chemie S.A. (La Jota, Barcelona, Spain).

**Determination of antimicrobial activity**

Agar disc diffusion method (Rios *et al.*, 1989; Piddock, 1990; Parish and Davidson, 1993; Collins *et al.*, 1995) was used to determine the antimicrobial activity of plant extracts. Twenty-five ml Muller-Hinton Agar (MHA) (Scharlau Microbiology) were poured in 9-cm Petri plates and allowed to solidify and dry. One hundred µl of 24 h incubated test microorganism of standard size ($10^6$ for bacteria and yeast, $10^5$ for mold) was spread on the surface of MHA and allowed to dry for 15 min. Four pieces of sterile 6-mm assay discs (Schleicher and Schuell) were then distributed on the surface of the agar. Two of the discs were impregnated with 40 µl of freeze-dried extract dissolved in sterile distilled water at 100,000 parts per million (ppm) concentration. One of remaining discs was impregnated with 40 µl 750-ppm tetracycline for bacteria or 625-ppm ketoconazole for yeast and mold as positive control while the other disc was impregnated with 40 µl sterile distilled water as negative control. The plates were then left at room temperature for 30 min to let the extract and controls diffuse to the agar and incubated at 37 ± 1°C for 20 h for bacteria or 30 ± 1°C for 5 d for yeast and mold. Plates were observed for inhibition zones (zones showing no microbial growth) around the discs after the incubation period.

**Determination of minimum inhibitory concentration (MIC)**

Since agar disc diffusion assay is a qualitative method used in antimicrobial testing, the determination of MIC was done to obtain quantitative results on the antibacterial and antimycotic effects of the plant extracts. The method used to determine the MIC was the agar dilution method of the European Society of Clinical Microbiology and Infectious Diseases (2000). Freeze-dried extract was dissolved in sterile distilled water in two-fold dilutions from 100 to 204,800 ppm. One ml of each of the extract dilutions was individually added to 19-ml MHA for bacteria or acidified PDA for yeast and mold maintained in water bath at 50°C and then poured into Petri plates giving final concentrations from 5 to 10,240 ppm. The final concentration was calculated from the following equation:

$$C_f = C_i / 20$$

where, $C_f$ is the final concentration of extract in the agar and $C_i$ is the initial concentration of extract in the solution.

Test microorganism was spotted on the surface of the solidified extract-agar mixture. Four spots were placed in each plate at an amount of 10 µl (ca $10^4$ cfu for bacteria and yeast or $10^3$ cfu for mold) per spot. The plates were inoculated by starting from the lowest concentration up to the highest concentration. Controls (agar without extract) were also inoculated at the start and at the end of the dilutions. After inoculation, plates were allowed to dry for 30 min. Plates were incubated at 37 ± 1°C for 18 h for bacteria or 30 ± 1°C for 5 d for yeast and mold. The lowest concentration which showed no visible growth of the microorganism was considered as the MIC for the extract.

## RESULTS AND DISCUSSION

### Determination of antimicrobial activity

Among the extracts used, DPB showed antimicrobial activity against all of the test microorganisms (Table 1). FPB was found to be active against *Staphylococcus aureus* and *Lactobacillus sp.* while DPN can only inhibit the growth of *Lactobacillus sp.* (Table 1).

Antimicrobial activity of extract from *Piper betle* Linn. could be attributed to its chemical constituents. *Piper betle* Linn. were found to contain phenolic compounds such as cavicol, cavibetol, carvacrol, eugenol and allilpyrocatechol (Farnsworth and Bunyapraphatsara, 1992). According to Katayama and Nagai (1960), as reported by Conner (1993), carvacrol, together with thymol, inhibits *Bacillus subtilis*, *Salmonella enteritidis*, *S. aureus, Pseudomonas aeruginosa*, *Pseudomonas morganii* and *Escherichia coli*. Eugenol is an essential oil known to be bacteriostatic and fungistatic (Beuchat and Golden, 1989; Cowan, 1999). Catechol, on the other hand, is a hydroxylated phenol which is toxic to microorganisms (Cowan, 1999). It is thought that enzyme inhibition by the phenolic compounds is the mechanism for microorganism inhibition. This is possibly done by the oxidized compounds through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999).

Same study on antimicrobial activity of *Piper betle* Linn. showed that yellow variety extract obtained from hot water extraction could inhibit *Bacillus stearothermorphilus* and kill *Pseudomonas fluorescens* and *P. aeruginosa*. This was revealed after studying the effect of extraction methods on the antimicrobial activity of two varieties of *Piper betle* Linn. using well diffusion and pour plate methods by Jenie *et al.* (2001).

The difference between the antimicrobial activity of FPB and DPB could be explained by the amount of antimicrobial substances present in each form. Drying the *Piper betle* Linn. leaves could have concentrated the antimicrobial compounds per amount of the extract as opposed to the antimicrobial compounds present in the same amount of the fresh *Piper betle* Linn. extract.

The sensitivity of *Lactobacillus sp.* to all of the plant extracts (FPB, DPB and DPN) could be

**Table 1** Antimicrobial activity of plant extracts (100,000 ppm) using agar disc diffusion assay[a].

| Test microorganism | FPB | DPB | DPN |
|---|---|---|---|
| *Escherichia coli* | - | + | - |
| *Staphylococcus aureus* | + | + | - |
| *Salmonella derby* | - | + | - |
| *Pseudomonas aeruginosa* | - | + | - |
| *Bacillus subtilis* | - | + | - |
| *Lactobacillus sp.* | + | + | + |
| *Saccharomyces cerevisiae* | - | + | - |
| *Aspergillus niger* | - | + | - |

[a] + means growth inhibition.
- means no growth inhibition.
FPB = Fresh *Piper betle* Linn.
DPB = Dried *Piper betle* Linn.
DPN = Dried *Phyllanthus niruri*

accounted for the presence of antimicrobial compounds common to the 3 extracts that had inhibitory effects on the microorganism. Identification of these compounds could be a subject of future study.

**Determination of Minimum Inhibitory Concentration (MIC)**

Dried *Piper betle* Linn. extract inhibited the growth of the test microorganisms with minimum concentrations ranging from 160-10,240 ppm as opposed to the FPB and DPN which did not show any inhibition of microbial growth at the highest concentration used (Table 2). Among the test microorganisms, *Saccharomyces cerevisiae* was the most sensitive to DPB which only required 160 ppm to inhibit its growth followed by *S. aureus* and *Aspergillus niger* which were inhibited by 640 ppm concentration (Table 2). The results are in accordance with the findings in disc diffusion assay. Only extract from DPB was inhibitory of all the foodborne spoilage and pathogenic microorganisms used in this study. The inhibition of *S. aureus* and *Lactobacillus sp*. by FPB in the disc diffusion assay was not evident in this part of the study because the highest concentration used (10,240 ppm) was much lower than the one used in the screening experiment which was 100,000 ppm. The same reason could also be used for the non-inhibition of *Lactobacillus sp*. by DPN.

Jenie *et al*. (2001) reported that the whole extract (mixture of volatile and nonvolatile extract) of *Piper betle* Linn. could inhibit *S. aureus* and *E. coli* at 0.025% (v/v) while Farnsworth and Bunyapraphatsara (1992) found that the MIC of *Piper betle* Linn. leaves against *S. aureus* were 0.25%. The computed values for DPB from this study revealed that *S. aureus* and *E. coli* could be inhibited with a minimum concentration of 0.064% (w/v) and 0.25% (w/v), respectively. These results can not be compared with the values obtained by the mentioned authors since different extraction and MIC determination methods were used.

## CONCLUSION

This study showed that crude ethanol extracts from medicinal plants could inhibit certain foodborne spoilage and pathogenic microorganisms. Extract from dried *Piper betle Linn.* inhibited all test microorganisms with minimum inhibition concentration ranging from 160 to 10,240 ppm. Results revealed that the extract can be used as source of natural

**Table 2** MIC (ppm) of plant extracts against test microorganisms.

| Test microorganism | FPB | DPB | DPN |
|---|---|---|---|
| *Escherichia coli* | > 10,240 | 2,560 | > 10,240 |
| *Staphylococcus aureus* | > 10,240 | 640 | > 10,240 |
| *Salmonella derby* | > 10,240 | 2,560 | > 10,240 |
| *Pseudomonas aeruginosa* | > 10,240 | 2,560 | > 10,240 |
| *Bacillus subtilis* | > 10,240 | 10,240 | > 10,240 |
| *Lactobacillus sp.* | > 10,240 | 2,560 | > 10,240 |
| *Saccharomyces cerevisiae* | > 10,240 | 160 | > 10,240 |
| *Aspergillus niger* | > 10,240 | 640 | > 10,240 |

FPB = Fresh *Piper betle* Linn.
DPB = Dried *Piper betle* Linn.
DPN = Dried *Phyllanthus niruri*.

antimicrobial compounds which can be applied to foods to prevent growth of undesirable microorganisms. In addition, with the advent of novel food preservation techniques, extract from dried *Piper betle* Linn. could prove useful in antimicrobial food packaging. Incorporation of the extract in food packaging materials and determination of its effect on the shelf-life of food products are some topics for further research.

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