

Antibacterial Activity of Guyabano, Ulasimang Bato, Sambong, and Tsaang Gubat Leaf Extracts against Common Drug-resistant Bacteria

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ABSTRACT

Background. Using plants as antimicrobials has long been a practice of traditional healers and validating these customs may lead to the discovery and development of useful herbal medicines.

Objective. This study aimed to determine the antibacterial activity of guyabano, tsaang gubat, sambong, and ulasimang bato against common pathogens.

Methods. Aqueous or alcoholic leaf extracts of the different medicinal plants were prepared. The solid agar dilution method was used to determine the MIC of guyabano, tsaang gubat, sambong, and ulasimang bato against common pathogens including *Staphylococcus aureus*, *Echerichia coli*, *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Shigella flexneri*.

Results. The alcoholic leaf extract of guyabano showed moderate activity against oxacillin-sensitive *S. aureus* with an MIC of 5-6.3 mg/mL. However, tsaang gubat did not exhibit any antibacterial activity for drug-resistant enteric organisms (*S. typhi*, *S. flexneri*, and *E. coli*) and *S. aureus* at a concentration of 25 mg/mL. Even at a concentration of 100mg/mL, ulasimang bato failed to show any antibacterial activity against drug-resistant *S. aureus*, *S. pneumoniae*, *H. influenzae*, *E. coli*, and *P. aeruginosa*. Sambong alcoholic extract had some antibacterial activity against penicillin-resistant *S. pneumoniae* with an MIC of 12.5 mg/mL.

Conclusions. Guyabano alcoholic leaf extract showed moderate antibacterial activity against oxacillin-sensitive *S. aureus*. Sambong alcoholic extract likewise exhibited inhibitory activity against *S. pneumoniae*. However, tsaang gubat and ulasimang bato aqueous extracts failed to show significant antibacterial activity for the pathogens tested.

Key Words: antibacterial activity, guyabano, *Anona muricata*, tsaang gubat, *Ehretia microphylla*, *Carmona retusa*, sambong, *Blumea balsamifera*, ulasimang bato, pansit-pansitan, *Peperomia pellucida*

INTRODUCTION

Bacterial infections remain to be an important cause of morbidity in the Philippines with respiratory, gastrointestinal and urinary tract infections still being very prevalent.¹ Antibiotics have been used to treat bacterial infections and are considered one of the most important discoveries of the modern era. Unfortunately, because of extensive abuse and misuse of antibiotics both in humans and animals, rates of antimicrobial resistance have been increasing. According to a WHO Report in 2014, the top 7 common bacteria that cause serious diseases with widespread resistance include *E.coli*, *S. aureus*, *H. influenzae*, *Shigella*, *P. aeruginosa*, non-typhoidal *Salmonella*, *Klebsiella*, and *N. gonorrhoea*.² In the Philippines, *Staphylococcus aureus*, *Pseudomonas aeruginosa*,

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and *Acinetobacter baumannii* were reported to have high rates of resistance to conventional antibiotics.³ What is being feared now is an occurrence of a post-antibiotic era, wherein present antibiotics are ineffective against common diseases. Thus the efforts for discovery and development of new antimicrobials are still necessary in the modern era. To add to this problem, few pharmaceutical companies have invested in the discovery of new classes of antibacterials in the last 3 decades.⁴ This may be due to the enormous expenditures incurred for investigating new drugs, but the return of investment are much smaller for antibiotics which are only prescribed for a short period of time, compared to medications for lifestyle-related and chronic diseases such as diabetes and hypertension which are taken for very long periods of time.

Plants as sources of antimicrobials is an old concept which dates back to traditional medical systems in numerous cultures.⁵ Herbal preparations have been used by traditional healers as antiseptics, as well as cures for pneumonia, diarrheas, and other bacterial infections. This may be a logical strategy since plants produce metabolites to protect themselves against microbes in the environment.³ Many modern day medicines and even antibiotics are also nature-derived or plant-based or plant-derived.⁴ Quinine and artemisinin are two success stories of antimalarial agents derived from the cinchona bark and *Artemisia annua*, respectively.⁶ Three types of antibacterials may be developed from plants: traditional antibacterials, inhibitors of multidrug resistance pumps, or compounds which target bacterial virulence.⁷

Four commonly used medicinal plants in the Philippines include guyabano, tsaang gubat, sambong, and ulasimang bato. Guyabano or *Annona muricata* L (family Annonaceae) is cultivated throughout the Philippines and is used by Filipino traditional healers for various ailments. The roots are used for anemia while the leaves are used for cough and colds, indigestion, dysentery, amebiasis, diarrhea, and various types of pain.⁷ In other tropical countries such as Indonesia, India, and Brazil, guyabano is also commonly used to treat infections such as pneumonia, urinary tract infection, and skin diseases.⁸ Tsaang gubat or *Ehretia*

microphylla Lam. (synonym *Carmona retusa* (Vahl) Masam) (family Boraginaceae) or commonly called Philippine tea or Fukien tea, is found in the Philippines, India, Southern China, Taiwan, and the Malay Peninsula. The leaves are frequently used by traditional healers as a decoction for various types of gastrointestinal ailments such as diarrhea, vomiting, abdominal pain as well as for other indications such as insomnia, fever, cough, colds, and muscle pain.⁷ Sambong or *Blumea balsamifera* L. (family Compositae/Asteraceae) has been proven as an effective treatment for kidney stones.⁹ In the Philippines, traditional healers recommend using sambong for various ailments including cough, fever, influenza, dysentery, sore throat, malaria, boils, infected umbilical cord, sore eyes, and tuberculosis.⁷ Ulasimang bato or *Peperomia pellucida* (L.) Kunth (family Piperaceae) is also known as pansit-pansitan, sinaw-sinaw, or tangon-tangon. In the Philippines and West Africa, the whole plant is used as a warm poultice for abscesses and boils.¹⁰ Other cultures use the leaf decoction to treat urinary tract infections, headache, fever, eczema, abdominal pains, and convulsions.¹¹

Many of the folkloric uses of these 4 medicinal plants include treatment for different types of bacterial infections. Validation of folkloric uses of these herbs is the first step to determine their potential to be developed as antibacterial herbal medicines. This study aimed to determine the antibacterial activity of guyabano, tsaang gubat, sambong, and ulasimang bato against common pathogens.

MATERIALS AND METHODS

Plant materials

The 4 medicinal plants were grown and cultivated by the agricultural team of the University of the Philippines at Los Baños who also identified and authenticated the samples (Table 1). The leaves were selected, with the dirt and foreign materials removed, and were delivered to the pharmacy team at the University of the Philippines Manila. Onsite garbling was performed, then the leaves were washed with clean tap water. The leaves were then placed on drying

Table 1. Philippine medicinal plants evaluated for antibacterial activity

Scientific name	Local name	Family	Type of extract	Microorganisms tested
<i>Annona muricata</i>	Guyabano	Annonaceae	Ethanol	<i>S. aureus</i> <i>E. coli</i>
<i>Ehretia microphylla</i> (syn. <i>Carmona retusa</i>)	Tsaang gubat	Boraginaceae	Aqueous	<i>S. aureus</i> <i>E. coli</i> <i>Shigella dysenteriae</i> <i>Salmonella typhi</i>
<i>Blumea balsamifera</i>	Sambong	Asteraceae/Compositae	Ethanol and Aqueous	<i>S. aureus</i> <i>E. coli</i> <i>H. influenzae</i> <i>P. aeruginosa</i>
<i>Peperomia pellucida</i>	Ulasimang bato	Piperaceae	Aqueous	<i>S. aureus</i> <i>E. coli</i> <i>H. influenzae</i> <i>P. aeruginosa</i>

beds with electric fans for 2 days at room temperature. The leaves were dried in an oven at 60°C until the moisture content was less than 10% and were milled into a fine powder of 0.0278 inches.²

Plant Extract Preparation

Plant extracts were prepared at the Department of Industrial Pharmacy, College of Pharmacy, University of the Philippines Manila. The plant extracts were prepared following a modified procedure from the Philippine Pharmacopeia.¹² For alcoholic extraction, ethanol was added to completely submerge the leaves at a ratio of 4:1 (ethanol: powdered leaves). The solution with the leaves was then macerated for 24 hours with frequent shaking for the first five (5) hours and then was allowed to stand for nineteen hours. The solution was filtered rapidly using Whatmann filter paper No. 1 and the filtrate was set aside. The residue was washed with ethanol and filtered again. All filtrates were combined and were subjected to freeze drying.

For aqueous extraction, distilled water was added at a ratio of 4:1 (water: powdered leaves). The solution was heated for 15 minutes to make a decoction then cooled and filtered using previously boiled cheese cloth. The residue was washed with distilled water. All filtrates were combined and subjected to secondary filtration using Whatmann filter paper No. 1. Final filtrate was lyophilized. Prior to the antibacterial screening, the extracts were rendered sterile by filtering through a 0.22 µm filter disk and stored at -20°C prior to use.

Originally both aqueous and ethanolic extracts of all 4 plants were planned to be tested. Unfortunately, due to time constraints and lack of plant material (it was also tested for other pharmacologic activities and toxicity studies), not all types of extracts were tested.

Test Microorganisms

The following common pathogens were used in the study: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and enteric organisms like *Escherichia coli*, *Salmonella typhi*, and *Shigella flexneri*. These organisms were chosen since they are the common bacteria that cause serious diseases with widespread resistance.

The isolates of *Streptococcus pneumoniae* and *Haemophilus influenzae* were obtained from the Department of Microbiology, Research Institute for Tropical Medicine (RITM). Biochemical tests were done on the isolates obtained from RITM to confirm the bacterial identity. The *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Shigella flexneri* clinical isolates were obtained from the Department of Medical Microbiology, College of Public Health, UP Manila and were tested for their antimicrobial susceptibility to also confirm their identities. Table 1 shows the microorganisms tested for each plant. Since tsaang gubat has been proven efficacious for abdominal colic due to gastroenteritis, evaluating for antibacterial activity against

gastrointestinal pathogens was deemed important thus, *Salmonella typhi* and *Shigella flexneri* were also tested.

Antibacterial assay of plant extracts

The plant extracts were tested for antibacterial activity by determining their minimum inhibitory concentrations (MIC). Agar dilution susceptibility method described by Turnidge and Bell¹³ was modified. Instead of commercially available pure antimicrobial powders, the leaf plant extracts were used. Ten concentrations levels (100,000 - 180 µg/mL) were tested, when possible, for each plant extract and its activity was tested against the selected bacteria. The different concentrations of extract were poured onto ten different petri dishes. The correct doses of antibiotic control and negative control (triple distilled water) were used. Mueller Hinton agar was prepared according to the manufacturer's instructions (Laboratorios, Conda SA, Madrid, Spain) and cooled to 50°C. Agar was added to each petri dish with either a dose of the plant extract, or the positive or negative control. The agar was allowed to set and the surfaces of the plates were allowed to dry for 10 minutes in a fan assisted drying cabinet without ultraviolet light.

Growth method was used for Enterobacteriaceae, *Pseudomonas*, *Streptococci*, *Haemophilus*, and *Staphylococci*. At least 4 morphologically similar colonies were transferred with a sterile loop to the Isosensitest Broth and incubated with shaking at 35-37°C until the visible turbidity is equal or greater than the 0.5 McFarland Standard.

Plate inoculation

A micropipettor was used in delivering 1-2 µL of bacterial suspension of inoculum onto the surface of the agar. The inoculums were allowed to be absorbed into the agar prior to incubation. Three replicates of each dilution were performed.

Incubation conditions

Conditions for incubation of the plates are shown in the following list:

Enterobacteriaceae 35-37°C in air for 18-20 h
Pseudomonas spp 35-37°C in air for 18-20 h
Staphylococci (other than tests on methicillin/oxacillin) 35-37°C in air for 18-20 h
Staphylococci tests on methicillin/oxacillin 30°C in air for 24 h
S. pneumoniae 35-37°C in 4-6% CO₂ in air for 18-20 h
Haemophilus spp. 35-37°C in 4-6% CO₂ in air for 18-20 h
Salmonella typhi 35°C in air for 16-18 h
Shigella flexneri 35-37°C in air for 18-24 h

Reading and Interpretation

The Minimum Inhibitory Concentration (MIC) for the extract against a specific bacterium was the lowest concentration of extract at which there was no visible growth of the organism in 24 hours. The antibiotic-free control

plate should have visible growth for the assay to be valid. The expected MIC was based on the breakpoints set by the European Committee on Antimicrobial Susceptibility Testing 2016 for the specific organism and antibiotic control. Strong antibacterial activity was defined as a MIC <5 mg/mL.¹⁴

RESULTS

Four Philippine medicinal plants were tested for their antibacterial activity through the agar dilution method. Guyabano was only tested for 2 representative organisms. It had moderate activity against *S. aureus* which was sensitive to oxacillin with an MIC of 5-6.3 mg/mL. For ceftriaxone-sensitive *E. coli*, it showed poor inhibitory activity for all concentrations tested up to the highest dose tested which was 15.8 mg/mL.

Tsaang gubat aqueous leaf extract was tested against common enteric bacteria (*Salmonella typhi*, *Shigella flexneri*, *E. coli*) and *S. aureus*. The maximum concentration tested was 25,000 µg/mL because the extract was thick and could not be filtered to be rendered sterile. There was significant growth of all bacteria tested (ampicillin-resistant *Salmonella typhi*, ampicillin-sensitive *Shigella flexneri*, ampicillin-resistant *E. coli* and methicillin-sensitive *S. aureus*) in all concentrations tested even at the maximum concentration of tsaang gubat extract.

Aqueous and alcoholic extracts of sambong were tested against all 5 resistant bacteria to a maximum concentration of 50,000 µg/mL due to the thickness of the extract (Table 2). The MICs ranged from 12.5 to >50 mg/mL, showing some antibacterial activity for the pathogens tested. *S. pneumoniae* had the lowest MIC using the alcoholic sambong extract.

Ulasimang bato aqueous leaf extract was tested for its antibacterial activity against resistant *S. aureus*, *S. pneumoniae*, *H. influenzae*, *E. coli*, and *Pseudomonas aeruginosa*. The ulasimang bato leaf extract did not show any antibacterial activity against both gram positive and gram negative bacteria used in the study. (Table 3).

DISCUSSION

There is a pressing need for new antibacterials and medicinal plants show a promising potential as source of antibacterial agents. Four common Philippine medicinal plants were tested for their antibacterial activity. Among the 4 plants, the guyabano alcoholic leaf extract showed some promise against *Staphylococcus aureus*, although this was a methicillin-sensitive strain. A few studies have shown diverse results. A study from Indonesia showed poorer antibacterial activity of guyabano methanol and chloroform leaf extract against *S. aureus* and *E. coli*, with the methanol extract having a zone of inhibition greater than 14mm at 150mg/mL for *S. aureus* and 250 mg/mL for *E. coli*.¹⁴ This study indicates that very high concentrations of the Indonesian guyabano

Table 2. MIC of Sambong aqueous and alcoholic extracts against common bacterial pathogens

Bacteria	MIC (mg/mL) for Aqueous extract	MIC (mg/mL) for Ethanol extract	Susceptibility of the bacteria tested
<i>S. aureus</i>	25	25	Resistant to vancomycin
<i>S. pneumoniae</i>	>50	12.5	Resistant to penicillin
<i>H. influenzae</i>	>50	25	Resistant to ceftriaxone
<i>E. coli</i>	>50	>50	Resistant to ampicillin
<i>P. aeruginosa</i>	>50	>50	Resistant to ceftazidime

Table 3. MIC of Ulasimang bato aqueous extract against common bacterial pathogens

Bacteria	MIC mg/mL	Susceptibility of the bacteria tested
<i>S. aureus</i>	>100	Bacteria resistant to vancomycin
<i>S. pneumoniae</i>	>100	Bacteria resistant to penicillin
<i>H. influenzae</i>	>100	Bacteria resistant to ampicillin and ceftriaxone
<i>E. coli</i>	>100	Bacteria resistant to ampicillin
<i>P. aeruginosa</i>	>100	Bacteria resistant to ceftazidime

leaf extracts are needed to produce antibacterial activity. Another study used the agar cup method in determining the antibacterial activity of *Annona muricata* leaf extracts. High concentrations ranging from 50-400 mg/mL were used.¹⁵ The methanolic leaf extract showed higher antibacterial activity against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* with zones of inhibition greater than 14 mm even at the lowest dose of 50 mg/mL.

In the present study, tsaang gubat was tested against several gastrointestinal bacterial pathogens. Unfortunately, at the highest dose tested (25mg/mL), the aqueous extract did not exhibit any antibacterial activity. A study from India also revealed that tsaang gubat did not have any antibacterial activity against *E. coli*, *Klebsiella aerogenes*, *Proteus vulgaris*, and *Pseudomonas aerogenes*.¹⁶ In contrast, another study on tsaang gubat from the Philippines showed that the ethanol extract had an MIC of 1.25 mg/mL for methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, and MIC of 2.5 mg/mL for *Pseudomonas aeruginosa*.¹⁷ In another study from India, the methanol and chloroform leaf extract of tsaang gubat showed large zones of inhibition (17-28 mm) against *Pseudomonas aeruginosa*, *K. pneumoniae*, *Shigella flexneri*, and *Bacillus subtilis*.¹⁸ Results of the present study on the antibacterial property of tsaang gubat may not have been as effective as the study from India due to the different extraction method. A second look at the ethanolic extract may be worth repeating to see its reproducibility for the Philippine variety.

Both the aqueous and ethanolic sambong leaf extracts were tested against several common bacterial pathogens. Some antibacterial activity was seen against vancomycin-resistant *S. aureus* with an MIC of 25 mg/mL and penicillin-resistant *S. pneumoniae* with an MIC of 12.5 mg/mL. Further studies may be conducted, such as those

for less resistant bacteria to determine if an antibacterial preparation from sambong is worth pursuing. Sakee also determined the antibacterial activity of sambong but used the essential oil and other types of extracts. It was found that the essential oil had the most potent activity compared to the other extracts with an MIC of 0.12 mg/mL against *S. aureus* and *C. albicans*.¹⁹

Results from the present study also revealed that the aqueous extract of ulasimang bato showed poor antibacterial activity for the resistant bacteria tested with MIC values higher than 100mg/mL. In contrast, another study showed low MICs for all bacteria studied. MIC values ranged between 0.031 mg/ml and 0.125 mg/mL. Bacteria included *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, and *Klebsiella* although the sensitivity to other antimicrobials was not mentioned in the study.²⁰ Differences in activities may be due to different varieties of plants or growth conditions leading to the production of different plant metabolites.

CONCLUSION

Among the 4 Philippine medicinal plants tested, guyabano aqueous leaf extract showed some promise as an antibacterial agent possibly for a topical indication with an MIC of 5 mg/mL for methicillin-sensitive *S. aureus*. The aqueous leaf extracts of 3 other plants (sambong, tsaang gubat, and ulasimang bato) exhibited poor activity for the resistant bacteria tested. However, the sambong alcoholic extract exhibited inhibitory activity against penicillin-resistant *S. pneumoniae*.

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Statement of authorship

All authors participated in drafting the protocol. Cavinta and Sara were involved in data collection. Maramba, Cavinta and Sara were involved in data analysis. All authors were involved in writing the manuscript and approved the final version submitted.

Authors disclosure

All authors declared no conflict of interest.

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