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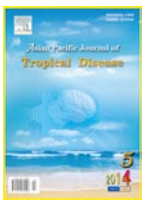

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# Antibacterial activity of crude ethanolic extract and solvent fractions of *Ficus pseudopalma* Blanco leaves

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PEER REVIEW

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**Comments**

The preliminary result of the study is relevant since it is the first to investigate the antibacterial properties of *F. pseudopalma* leaf extract and opened the need to further characterize the bioactive compounds present. Furthermore, the result of the study also suggests that the above mentioned ornamental tree can be a potential source in developing effective drugs against disease causing Gram-positive bacteria.
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ABSTRACT

**Objective:** To investigate the antimicrobial properties of *Ficus pseudopalma* (*F. pseudopalma*) leaf extracts.
**Methods:** The antibacterial properties of *F. pseudopalma* Blanco crude ethanolic leaf extract, and its solvent fractions chloroform (CF), ethylacetate (EF) and water fractions were evaluated through antibacterial agar disc diffusion method and the minimum inhibitory concentration (MIC) was determined. Five Gram-positive and five Gram-negative bacteria were used for the study.
**Results:** The zone of inhibitions obtained from the antibacterial agar diffusion disc method showed that CF, and EF exhibited active (14-19 mm) antibacterial activity against *Bacillus subtilis* UST CMS 1011, and partially active (10-13 mm) antibacterial properties against both *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228. Water exhibited no antibacterial properties against all microorganisms tested. The MIC values observed for all Gram-positive bacteria tested were >5 mg/mL, except for *Bacillus subtilis* whose MIC value was 5 mg/mL for CF and EF fractions. All extracts exhibited no antibacterial activity against Gram-negative bacteria.
**Conclusions:** From this study, it can be concluded that *F. pseudopalma* extracts may be a potential antibacterial agent against Gram-positive bacteria. The antibacterial property may be attributed to flavonoids and terpenoids present in the crude ethanolic extract, CF and EF.

KEYWORDS

*Ficus pseudopalma* Blanco, Antibacterial activity, Phytochemicals

## 1. Introduction

Infectious diseases pose a serious health concern worldwide[1]. The development of drug-resistant pathogens due to indiscriminate use of antibiotics has compounded the need for new sources of antimicrobial agent.

Plants are a good source of natural products that may have potential antimicrobial properties. In developing countries, like the Philippines, medicinal plants have been used as an alternative in the management of infectious diseases where treatment and medicine may be too expensive or are unavailable. The genus *Ficus*, belonging to family Moraceae, has been widely documented to have diverse biological activities such as antioxidant, anticancer,

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antimutagenic, reno-protective, antibacterial, *etc*[2-8].

*Ficus pseudopalma* (*F. pseudopalma*) Blanco is an endemic medicinal and ornamental tree found throughout the Philippines. The folkloric practice of using fig leaves to decoct for the treatment of kidney stones and diabetes is recently validated by a study on the antioxidant and antiurolithic properties of *F. pseudopalma* leaves[2,9]. The dichloromethane extract of the air-dried leaves of *F. pseudopalma* afforded squalene, polyprenol, β-amyrin fatty acid ester, α-amyrin acetate and β-amyrin acetate, lupeol fatty acid ester, lupenone, oleane, and ursenone[10]. Despite recent studies are on the biological activities of *F. pseudopalma* leaf extracts, its antimicrobial properties have not yet been investigated. Thus, this study aims to assess the antibacterial potential of *F. pseudopalma* crude ethanolic leaf extracts and its fractions against selected Gram-negative and Gram-positive bacteria.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Technical grade 95% ethanol, analytical grade chloroform, ethyl acetate (RCI Labscan Ltd., Thailand) and *p*-iodonitrotetrazolium chloride (Sigma-Aldrich Chemical Co., Singapore) were purchased from Belman Laboratories. Mueller Hinton agar (MHA) were obtained from Becton Dickinson, Heidelberg. The reference antibiotic susceptibility discs gentamicin and tetracycline (mastdiscs™ AST, Mast Group Ltd., UK) were purchased from Medical Test Systems, Inc.

### 2.2. Plant material

Matured leaves of *F. pseudopalma* used in this study were collected in May 2012 from Umali Subdivision, Los Banos, Laguna, Philippines. It was authenticated at the Botany Division, National Museum, Philippines.

### 2.3. Plant preparation and extraction

About 500 g of air-dried, pulverized and sieved *F. pseudopalma* leaves were percolated with 95% ethanol for 2 d. The collected extract was concentrated to dryness using a rotary evaporator (Eyela, USA) at 40 °C to yield 3.23% of the crude ethanolic extract. The crude ethanolic extract (10 g) was sequentially partitioned with chloroform (CF), ethylacetate (EF), and water (WF) to yield the following fractions: CF, EF and WF fractions. These fractions were evaporated to dryness by reduced-pressure evaporation at 40 °C in a rotary evaporator (Eyela, USA). The crude extract and its fractions were stored in tightly sealed collection bottles at −20 °C until use.

### 2.4. Preparation of stock and sample solution

Stock solutions of crude ethanolic extract and its fractions CF, EF and WF at 100 mg/mL concentration were prepared using dimethyl sulfoxide (DMSO) as solvent. For the antibacterial minimum inhibitory concentration (MIC) assay, the stock solutions were serially diluted with Mueller Hinton broth (MHB) containing 1% Tween 20 to obtain concentrations between to 62.5 and 5 000 μg/mL. All extracts were filtered with 0.22 μm syringe filters (Minisart, Sartorius) prior to use.

### 2.5. Bacterial culture

The following bacterial strains were obtained from the Univeristy of Santo Tomas Collection of Microbial Culture (USTCMS): Gram-negative bacteria [*Escherichia coli* ATCC 25922 (*E. coli*), *Escherichia hormaechei* ATCC 700323, *Klebsiella oxytoca* ATCC 7003224, *Klebsiella pneumonia* USTCMS 1040 (*K. pneumonia*) and *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*)] and Gram-positive bacteria [*Staphylococcus aureus* ATCC 25923 and ATCC 29213 (*S. aureus*), *Staphylococcus sciuri* ATCC 29061, *Staphylococcus epidermidis* ATCC 12228 and *Bacillus subtilis* USTCMS 1011 (*B. subtilis*)]. The bacterial cultures were maintained on MHA slant at 4 °C, and were subcultured on fresh agar plate for 24 h before the antibacterial assay.

### 2.6. Preparation of inoculum

A loopful of bacteria was inoculated on MHB and was incubated for 24 h at 36 °C. Then, the turbidity of the bacterial suspension was adjusted to 0.5 McFarland turbidity standard, and is equivalent to approximately $1.5\times10^8$ CFU/mL. This was used for the standardization of the antibacterial assay.

### 2.7. Preparation of discs

Whatman filter paper No. 1 discs of 6 mm diameter were sterilized using hot air oven at 160 °C for 1 h. Then 20 μL of the test extract containing 100 mg/mL was introduced on the sterile paper discs, and the solvents were allowed to evaporate on a stream of air. The solvents used for dissolving the extracts served as negative controls, while reference antibiotics gentamicin (GM 10C; 10 μg) and tetracycline (T 10C; 10 μg) were used as positive control for Gram-negative bacteria and Gram-positive bacteria, respectively.

### 2.8. Antibacterial agar disc diffusion assay: Kirby-Bauer method

About 15 mL of MHA (Becton Dickinson, Heidelberg) was poured in sterile 100 mm Petri dish, and was allowed to solidify. Then, 0.5 mL of the bacterial inoculum containing $1.5\times10^8$ CFU/mL was flooded on the surface of the MHA plates and was spread all over three times by rotating the plate 60° after each streaking using a sterile cotton applicator. Subsequently, filter paper discs impregnated with the test extracts and negative controls, as well as the reference antibiotic discs were placed on the surface of the agar at equidistant points using sterile forceps. The plates were incubated at 36 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition to the nearest millimeter around the disc using a vernier caliper. The tests were carried out in triplicate,

**Table 1**

Antibacterial activites (in mm) of *F. psendopalma* Blanco extracts and controls on bacteria as determined by antibacterial agar disc diffusion assay.

| Name of microorganism | Crude ethanolic extract | CF | EF | WF | Tetracyclin | Gentamycin | DMSO |
|---|---|---|---|---|---|---|---|
| Gram-positive bacteria | | | | | | | |
| *S. aureus* | 13.39±1.12 | 13.31±0.75 | 15.82±0.65 | 6.00±0.00 | 39.22±0.53 | nd | 6.00±0.0 |
| *S. sciuri* | 9.33±0.67 | 10.19±3.12 | 8.67±0.91 | 6.00±0.00 | 33.31±1.72 | nd | 6.00±0.0 |
| *S. aureus* | 6.49±0.49 | 5.58±2.58 | 14.16±0.11 | 6.00±0.00 | 35.75±0.02 | nd | 6.00±0.0 |
| *S. epidermidis* | 13.34±1.03 | 11.61±1.15 | 12.40±0.21 | 9.22±3.22 | 33.87±0.33 | nd | 6.00±0.0 |
| *B. subtilis* | 16.41±1.70 | 16.04±1.25 | 16.88±0.42 | 6.00±0.00 | 22.68±0.87 | nd | 6.00±0.0 |
| Gram-negative bacteria | | | | | | | |
| *E. horm-aechei* | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | nd | 19.66±1.38 | 6.00±0.0 |
| *K. oxytoca* | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | nd | 18.31±1.16 | 6.00±0.0 |
| *K. pneumonia* | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | nd | 18.95±1.59 | 6.00±0.0 |
| *P. aeruginosa* | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | nd | 20.94±0.34 | 6.00±0.0 |
| *E. coli* | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | nd | 17.89±0.73 | 6.00±0.0 |

Zone of Inhibition was determined by antibacterial agar disc diffusion assay. Results were presented as mean±SEM. Data show the partially active to active antibacterial activities of the crude extract and CF and EF fractions against Gram-positive bacteria. nd: not determined; CF: chloroform fraction; EF: ethyacetate fraction; WF: water fraction. Zone of Inhibition <10 mm: inactive; 10-13 mm: partially active; 14-19 mm: active; >19 mm: very active antibacterial activities[13].

and results were recorded as mean±SD. Zones of inhibitions greater than 6 mm were considered susceptible to the extracts[11] such that those measuring <10 mm were classified as inactive, 10-13 mm as partially active, 14-19 mm as active, and >19 mm as very active antibacterial activities[12].

### 2.9. MIC

The MICs of the ethanolic extract, fractions and reference antibiotic were determined for bacterial cultures that are susceptible to the extracts. About 95 μL of MHB and 5 μL of inoculum containing $1.5\times10^6$ CFU/mL was pipetted into designated wells of the 96-well microtiter plate, except for the negative control wells consisting of 100 μL of MHB. A volume of 100 μL of the extract at varying concentrations ranging of 5 000 to 62.5 μg/mL was added to its designated wells. The plates were covered with a sterile plate sealer, and were incubated at 30 °C for 24h. Forty microliter of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (Sigma-Aldrich, Singapore) was added into its designated wells. The plate was incubated again at 36 °C for 30 min. Viable bacteria reduced the yellow dye to a pink color. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth. This was done in triplicates[11].

### 2.10. Statistical analysis

Zone of inhibitions were expressed as mean±SEM of three trials in millimetres.

## 3. Results

*F. pseudopalma* leaf extracts exhibited inhibitory activities against Gram-positive bacteria. The *in vitro* antibacterial agar disc diffusion assay showed the inhibitory activities of *F. pseudopalma* crude ethanolic leaf extract and its CF and EF fractions against most Gram-positive bacteria, while having no antibacterial activity against all Gram-negative bacteria tested. Furthermore, WF showed no inhibitory activity against all bacteria tested.

Among all bacteria tested, results showed that *B. subtilis* USTCMS 1011 was highly susceptible to *F. pseudopalma* crude ethanolic extract and its CF and EF fraction, while EF had an active antibacterial activity against both *S. aureus* ATCC 25923 (15.82±0.65) and *S. aureus* ATCC 29213 (14.16±0.11). On the otherhand, crude ethanolic extract, CF and EF fractions were found to be partially active against *S. epididermidis* ATCC 12228. The crude ethanolic extract and CF fraction showed partially active antibacterial activities against *S. aureus* ATCC 25923. Partially active antibacterial activity was also demonstrated by fraction CF against *S. scuiri* ATCC 29061. Both positive controls gentamicin and tetracycline still had the greatest inhibitory activity against all bacteria tested, while the negative control DMSO had no antibacterial activity against all bacteria tested (Table 1).

The crude extract and its CF and EF fractions that showed antibacterial activities against Gram-positive bacteria were evaluated for its MIC. Results reveal a weak antibacterial activity against the bacteria tested since the MIC obtained for all extracts was >5 mg/mL, except for *B. subtilis* USTCMS 1011 whose MIC is 5 mg/mL for CF and EF fractions (Table 2).

**Table 2**

Minimum Inhibitory concentration of *F. pseudopalma* Blanco extracts against Gram-positive bactria.

| Gram-positive bacteria | Crude ethanolic extract | Chloroform fraction | Ethyl acetate fraction |
|---|---|---|---|
| *S. aureus* | >5 | >5 | >5 |
| *S. sciuri* | >5 | >5 | >5 |
| *S. aureus* | >5 | >5 | >5 |
| *S. epidermidis* | >5 | >5 | >5 |
| *B. subtilis* | >5 | 5 | 5 |

*F. pseudopalma* Blanco leaf extracts tested for MIC were crude ethanolic extract. Data reveals a weak antibacterial activity since the lowest MIC value determined was 5 mg/mL.

## 4. Discussion

This was the first study to investigate the antibacterial activities of *F. pseudopalma* leaf extracts against Gram-positive and Gram-negative bacteria. The antibacterial agar disc diffusion assay demonstrated the inhibitory activities of *F. pseudopalma* crude ethanolic extracts and its CF and EF fractions against selected Gram-positive bacteria, whereby the most active inhibitory activities were against *B. subtilis*

USTCMS 1011.

The antimicrobial activities of *Ficus* species have been well documented in traditional medicine. Contemporary studies on the use of *Ficus carica* (*F. carica*) ethy acetate latex extract against *Candida albicans* (*C. albicans*), *Enterococcus faecalis, Citobacter freudei, P. aeruginosa, E. coli*, and *Proteus mirabilis*[13], and the antibacterial property of *F. carica* against oral bacteria[14] has been reported. The methanolic extract of *Ficus vasta* exhibited antibacterial activity against *S. aureus* at 50 μg/mL[15]. The methanol extract of *Ficus exasperata* exhibited a wide range of activity on *P. aeruginosa, Salmonella typhi, S. aureus, E. coli* and *K. pneumonia*[16], while its aqueous and ethanolic leaf extracts inhibited the growth of *P. aeroginosa, Salmonella typhii, Enterococcus faecalis* and *E. coli*[17]. Phytochemical analysis revealed the presence of saponins, tannins, steroids and phlobatannins in the tested plant parts[16,17].

Phytochemical screening on the dichloromethane leaf extract of *F. pseudopalma* revealed the presence of squalene, polyprenol, β-amyrin fatty acid ester, α-amyrin acetate and β-amyrin acetate, lupeol fatty acid ester, lupenone, oleanone, and ursenone[10]. The presence of lupeol, a pentacyclic triterpene, was identified in the acetone:methanol fraction from the crude ethanolic leaf extract[18], in the crude ethanolic leaf extract and in its chloroform and ethylacetate fractions[19] and in the ethyl acetate fraction from the crude dichloromethane extract[2]of *F. pseudopalma* through HPLC analysis. The presence of flavonoids and the triterpene lupeol may be responsible for the antibacterial activities of *F. pseudopalma* leaf extracts.

Earlier studies revealed that alkaloids, flavonoids, and terpenoids isolated from various *Ficus* species were responsible for its antimicrobial activities. The alkaloids, *Ficus* eptine and antofine, from the methanolic extract of *Ficus septica* leaves displayed strong antibacterial and anti-fungal activities which supports the traditional use of the plant against fungal and bacterial infections[20]. In the study of Vital *et al*.[8], the ethanolic leaf extract of *F. septica* inhibited the growth of *E. coli*, *S. aureus*, and *C. albicans*.

Compounds with antimicrobial activities have been isolated from *Ficus ovata* stem bark. Terpenoids namely 3-friedelanone, betulinic acid and oleanoic acid have been shown to inhibit microbial growth probably through disruption of microbial membrane. Isoflavonoids 2-hydroxyisoprunetin and 6,7 (2-isopropenyl furo)-5,2′,4′-trihydroxyisoflavone actively inhibited the growth of *E. coli, K. pneumonia, P. aeruginosa, S. typhii, Citrobacter freundii, C. albicans* and *Microsporum audounii*. This may be due to the ability of isoflavonoids to form a complex with bacterial cell walls. The antibacterial activity of phenolic acid protocatechuic acid against Gram-positive and Gram-negative bacteria was also demonstrated[1].

Compounds luteolin and epiafzelechin from isolated *F. chlamydocarpa* and *F. cordata*, and laburnetin, alpinumisoflavone and genistein isolated from *F. chlamydocarpa* were able to inhibit the growth of 87.5% of the 16 microorganisms tested[11]. Quercetin and naringenin isolated from *Ficus benjamina* Linn. showed strong antibacterial activities against *Bacilus cereus* and *P. aeruginosa*[21]. In another study, quercetin was also found to be present in the crude ethanolic leaf extract and chloroform fraction of *F. pseudopalma*[19].

Lupeol isolated from *F. pseudopalma* leaf extracts have known antibacterial property[15]. This may be due to the ability of terpenoids to disrupt the formation of bacteria cell walls thereby inhibiting bacterial growth[1,22,23]. Lupeol have several reported biological activities such as antioxidant, cardioprotective, hepatoprotective, and anticancer properties; thereby making it a significant phytochemical identified from this plant[2,18,19,24].

In conclusion, the antibacterial activity of the crude extract and CF and EF fractions of *F. pseudopalma* leaves against Gram-positive bacteria tested may be attributed to the presence of flavonoids and terpenoids. The most susceptible Gram-positive organism was *B. subtilis*. This study may pave way for the potential use of *F. pseudopalma* as a new source of effective and affordable antibacterial compounds. Further studies on the phytochemical elucidation and characterization of bioactive compounds of this plant are recommended.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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## Comments

### *Background*

Bacterial pathogens exhibit antimicrobial resistance and remain to be a major threat on public health worldwide. Development of antimicrobial agents is therefore necessary to combat antimicrobial resistant pathogens. In this study the *F. pseudopalma* crude ethanolic leaf extract and its solvent fractions such as the CF, EF and WF were assessed against different strains of Gram-positive and Gram-negative bacteria.

### *Research frontiers*

The antimicrobial properties of *F. pseudopalma* leaf extracts have not yet been investigated. Therefore, this study was relevant because it assessed the antimicrobial properties of *F. pseudopalma* crude ethanolic leaf extract and its solvent fractions against different strains of Gram-positive and Gram-negative bacteria.

### *Related reports*

The results of the current study is in agreement with the previous study on *in-vitro* antimicrobial activity, however using methanolic extract of *F. carica* which showed that the methanolic extract was proven to be more effective against *Bacillus megaterium* among Gram-positive strains. Al-Yousuf studied the antibacterial activity of *F. carica* extract against six bacterial strains.

### *Innovations & breakthroughs*

The ethanolic leaf extracts and the chloroform and ethyl acetate solvent fractions of *F. pseudopalma* showed antimicrobial activities against different strains of Gram-positive bacteria, and therefore can used to develop potential antibacterial drugs against Gram-positive bacteria.

### *Applications*

The results of the study suggest that the *F. pseudopalma* ethanolic leaf extracts, chloroform fraction and ethyl acetate fraction showed antibacterial activities against Gram-positive bacteria indicating that this ornamental tree can be used in the formulation and development of effective drugs against disease causing Gram-positive bacteria.

### *Peer review*

The preliminary result of the study is relevant since it is the first to investigate the antibacterial properties of *F. pseudopalma* leaf extract and opened the need to further characterize the bioactive compounds present. Furthermore, the result of the study also suggests that the above mentioned ornamental tree can be a potential source in developing effective drugs against disease causing Gram-positive bacteria.

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