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Antibacterial Activity of Santan (*Ixora coccinea*) Leaf, Cacao (*Theobroma cacao*) Pod Husk, and Betel Palm (*Areca catechu*) Seed Extracts Against *Staphylococcus aureus*

Andrhea Lozada, Guenevere Damasin, Angelou Perez, Angelica Villasan, Alexis Tomboc, Angel Pescador, Regie Hubilla, Raven Gamo, H. Gelacio, Kry Nathalie France Tabid, Angelika Ysabelle Clapano, Jomila Buar, Julea Somera, Maika Garcia, Suzane Abayon, Geraldyn Cullo, Audra Leigh Galon, Jean Claude, Philippe Jackson, Karolyn Mae Jarrell, Sharina Melog, Angelu Shareen Okyo, Denny Rey Piscol, Jheri Mae Villanueva, Joseph Dave Pregoner *

University of the Immaculate Conception, Davao City, Davao del Sur, Philippines; lozadaandrhea@gmail.com (A.L.); Guenevere09@gmail.com (G.D.); angelousolasperez@gmail.com (A.P.); amarievillasan@gmail.com (A.V.); alexischriztenetomboc@gmail.com (A.T.); Cyruspescador@gmail.com (A.P.); Regieboyhubilla@gmail.com (R.H.); ravenphoebe18@gmail.com (R.G.); hgelacip@gmail.com (H.G.); krytabid27@gmail.com (K.N.F.T.); clapanoangelikaysabelle@gmail.com (A.Y.C.); jooomilabuar@gmail.com (J.B.); adinglessandra15@gmail.com (J.S.); Maika18garcia24@gmail.com (M.G.); Abayonsuzanemae@gmail.com (S.A.); geraldyncullo27@gmail.com (G.C.); audraleighgalon@gmail.com (A.L.G.); jeanclaude@gmail.com (J.C.); jacksonjcp10@gmail.com (P.J.); jarrellkarolynmae@gmail.com (K.M.J.); sharinamelog.new@gmail.com (S.M.); Gelayokyo@gmail.com (A.S.O.); piscoldennyrey@gmail.com (D.R.P.); vjherimae@gmail.com (J.M.V.)
* Correspondence: jpregoner@uic.edu.ph

ABSTRACT

The challenge of finding organic sources of antibacterial activity that are widely available, environment-friendly, and cost-effective with significant antibacterial efficacy, remains persistent especially in developing countries such as the Philippines. In this study, the antibacterial activity of Santan (*Ixora coccinea*) leaf, Cacao (*Theobroma cacao*) pod husk, and Betel Palm (*Areca catechu*) seed extracts against *Staphylococcus aureus* was assessed. Plant extract concentrations of 25%, 50%, 75%, and 100% were prepared and their antibacterial activity on *S. aureus* was assessed using the Kirby-Bauer disk diffusion susceptibility test. Antibacterial activities were determined using the zone of inhibition diameter. Distilled water and Penicillin were used as negative and positive controls, respectively. Results showed that Santan (*I. coccinea*) leaf extract with 75% concentration exhibited the highest antibacterial activity on *S. aureus* among all plant extracts used. However, its antibacterial activity was comparatively lesser than that of Penicillin. No antibacterial activity of Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seeds

extracts on *S. aureus* was observed. The differences in the zone of inhibition diameter in all extracts on *S. aureus* were significant ($p<0.01$).

KEYWORDS: antibacterial; Betel Palm; Cacao; Santan; *Staphylococcus aureus*

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1. Introduction

A substantial challenge in the worldwide healthcare is the need for effective and affordable medicines in preventing and treating bacterial infectious diseases [1,2]. The challenge remains persistent especially in developing countries such as India, Thailand, and Philippines [3,4]. Currently, there is a shift towards finding organic sources of antibacterial activity that are widely available, environment-friendly, and cost-effective with significant antibacterial efficacy [5,6].

Plants have been shown to produce a broad diversity of biomolecules and to contain anti-microbial substances such as glycosides, saponins, steroids, flavonoids, and alkaloids [7,8]. In the Philippines, three of the plants that are locally within reach are Santan (*I. coccinea*), Cacao (*T. cacao*) and Betel Palm (*A. catechu*). Existing literature shows that these plants, notably their leaves and seeds, have rich antibacterial properties that fight off bacteria present in the mouth of humans, as well as antioxidant, anti-inflammatory, anti-carcinogenic, immune-modulatory, vasodilatory and analgesic properties [9-13]. One significant stride in medical research is the conversion of plant industry waste by-products to medicinally important technology [14,15]. Being one of the best countries to grow cacao, the Philippines has been producing chocolate made with premium cacao beans. Cacao pod husks, the by-products of cacao plant, have demonstrated weak-to-moderate antimicrobial activity against *S. aureus* [16-18].

Presently, no existing research in the Philippines has been conducted to determine the antibacterial properties of these plants. Environmental factors and soil properties affect the plant characteristics, especially the plant's phytochemical substances [19-23]. Therefore, there is a need to determine the antibacterial activities of the plants in a different environment with variable soil properties to confirm findings of prior studies and for medical use in the Philippine setting.

One of the most extensively studied microorganisms in medicine is *S. aureus*, a major infectious pathogen that could cause a vast number of diseases. Bacterial analysis revealed that this gram-positive, round-shaped bacterium is found in normal human flora, located on the skin and mucous membranes, that if allowed to enter the internal tissues or bloodstream, may cause potentially serious infections that are common both in community-acquired as well as hospital-acquired settings [24]. *S. aureus* bacteremia is the leading cause of death in hospitalized patients with an incidence rate ranging 20 to 50 cases per 100,000 population per year [25]. Epidemiological studies found out that this bacterium was involved in 0.1% of all deaths and 0.2% of all hospital deaths [26].

Statistics of deaths from *S. aureus* comparatively accounts for a greater number of deaths than for AIDS, viral hepatitis and tuberculosis combined [27]. In the United States of America, invasive infections caused by this bacterium occurred in 94,000 people each year causing 19,000 deaths [28]. In the Philippines, an alarming 38.1% rate of *S. aureus* infection was reported [29]. The data from a multinational study revealed that the proportion of *S. aureus* infection among clinical isolates reached to a daunting rate of 59% in the Philippines [30]. In Philippine General Hospital, there is a 37.5% prevalence rate of the infection among pediatric patients with mortality rate ranging from 16% to 42% [31]. Infections with *S. aureus*, including Methicillin-resistant *S. aureus* (MRSA), can be treated with appropriate antibiotics, although the resistance to available antibiotics is increasing [32,33]. Hence, it is imperative to identify novel alternative medicines for treatment of *S. aureus*. This study was performed to analyze the antibacterial activity of Santan (*I. coccinea*) leaf, Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seed ethanolic extracts against *S. aureus*.

2. Materials and Methods

2.1 Study Setting

The experiment was conducted at the University of the Immaculate Conception, Davao City, Philippines. The plant extraction was carried out at the Physics Laboratory of Bonifacio Campus of the institution. The Kirby-Bauer disk diffusion susceptibility test was conducted at the Clinical Laboratory and Testing Center of the same campus.

2.2 Collection of Plant Materials

Santan (*I. coccinea*), Cacao (*T. cacao*), and ripe fruits of Betel Palm (*A. catechu*) were taken from local plant nurseries in Davao City, Philippines. These local plant nurseries are producing organically grown trees and shrub. The taxonomic classification of the plant materials was identified, certified, and verified by the Pharmacy/Chemistry Program of the University of the Immaculate Conception, Davao City, Philippines. After procurement and taxonomic classification, the leaves of Santan (*I. coccinea*) and pod husk of Cacao (*T. cacao*) were separated from the stem and roots of the collected plant materials. Ripe fruits of Betel Palm (*A. catechu*) were hammered using mortar and pestle to remove the seeds easily from the hard coating. The obtained leaves, pod husks, and seeds were then washed with distilled water to remove any debris. These were air dried at room temperature for several days until they became brittle and moisture-free. The plant materials were inspected daily until optimum dryness was achieved.

2.3 Preparation of Extract

Collected dried plant materials were ground using a house blender and the extracted materials were then subjected to the extraction process. The extraction process followed the standard procedures described in previous studies [34-36].

Dried Santan (*I. coccinea*) leaves were weighed at 1000 g, placed in an Erlenmeyer flask and treated with 650 mL of 95% ethanol. For Cacao (*T. cacao*), a total of 500 grams of powdered pod husk were weighed and deluged with 800 mL of 95% of ethanol inside an air-tight container. Meanwhile, 550 grams of the Betel Palm (*A. catechu*) seeds was weighed and macerated with 800 mL of 95% ethanol placed in an Erlenmeyer flask. The containers of all mixtures were stoppered, and the plant materials were kept soaked for 24 hours.

Whatman filter paper Number 1 was used to filter all mixtures. The obtained filtrates were concentrated by rotary evaporator at temperatures below 50°C to about 10 mL. The concentration of the obtained extracts was recorded as grams of dried plant material per mL of the extract obtained. Plant extract concentrations of 25%, 50%, 75%, and 100% were prepared. The container was appropriately labeled with the plant name, plant extract concentration and extraction date. The extract was stored with a tight stopper in the cold, at temperatures between 0°-5°C.

2.4 Infection Control Techniques

Before handling the selected microorganism, the researchers followed proper protocols to ensure no accidental contamination occurred during and after the study. The researchers disinfected the laboratory area using not less than 70% ethanol and bleach before and after the experiment were conducted. Proper personal protective equipment (PPE) was worn throughout the experiment: surgical/rubber gloves, face mask and laboratory gown. All equipment was sterilized using the autoclave before and after each use. Excess microorganism inoculums were destroyed using the autoclave before disposing in the designated collection site in the laboratory.

2.5 Preparation of Culture Media

The preparation of the agar followed the standardized procedures [37]. Medium was prepared in a beaker, 7 grams of plate count agar with 300 mL distilled water was poured down in the beaker. Media containing agar was heated to dissolve the agar before autoclaving. The medium was brought to boil using the hot plate and stirred constantly. The resulting solution was clear, lightly yellow with no undissolved materials. The solution was allowed to cool, and the mouth of flask was covered tightly with a cotton plug wrapped with aluminum foil and finally sealed with an autoclave indicator tape. The solution was sterilized in the autoclave at 121°C for 20 minutes. The culture media were aseptically dispensed into Petri dishes. About 20 mL was poured in the Petri dishes. The solution was then allowed to cool down further until it solidified. To avoid loss of moisture, the agar plates were stored away from light at 2-8°C in sealed containers.

2.6 Inoculation Procedure

The inoculation procedure followed the standardized steps outlined in previous literature [37,38]. The inoculating loop was heated over direct flame until it glowed red.

The loop was allowed to cool before getting a loopful of bacteria from a culture broth. The prepared culture media were held with the hand. The other hand inoculated the loopful of *S. aureus* using streak technique on one side of the plate. The loop was removed and the plate was closed. The plate was then turned by a quarter (90°) and the loop was heated again over direct flame and allowed to cool before inserting it again into the plate. Another streak was done and the procedure was repeated until creating four streaks. The last streak did not overlap with the first one. The plate was properly wrapped, inverted and incubated for 24 h at 37°C.

2.7 Kirby-Bauer Disk Diffusion Susceptibility Test

The antibacterial activity of the plant samples on *S. aureus* was analyzed using the Kirby-Bauer disk diffusion susceptibility test. The steps were described in previous research [39,40]. The bottom of the agar plate was marked with six equidistant spots. Using a puncher, filter paper disks with 6 mm diameter were made, put in a Petri dish and autoclaved. With sterile forceps, the filter paper disks were dipped in various plant extracts with 25%, 50%, 75%, and 100% concentration and placed in corresponding spots. Using sterile forceps, one filter paper disk was dipped in distilled water while another disk was dipped in Penicillin solution. These negative and positive controls were placed on the remaining spots. The Petri dish was wrapped with aluminum foil and incubated at 37°C for 18-24 h. Kirby-Bauer disk diffusion susceptibility test was carried out in three trials. The clear zones (zone of inhibition) were observed around the paper disks and the diameter was measured using a ruler or caliper.

2.8 Data Analysis

The outcome variable of this study was the zone of inhibition of *S. aureus*. This was measured using the scale as follows: (1) <10.0 mm zone of inhibition means that the plant extract has an inactive antibacterial action on *S. aureus*; (2) 10.0–13.0 mm zone of inhibition means that the plant extract has a partially active antibacterial action on *S. aureus*; (3) 14.0–19.0 mm zone of inhibition means that the plant extract has an active antibacterial action on *S. aureus*; (4) >19.0 mm zone of inhibition means that the plant extract has a very active antibacterial action on *S. aureus* [34].

Descriptive and inferential statistics were used for the analysis. Zone of inhibition diameter was expressed as mean ± standard deviation. One-Way Analysis of Variance (ANOVA) was used to determine the significance of *S. aureus* zone of inhibition diameter difference among controls and plant extract concentrations used. A p-value below 0.01 is considered significant.

3. Results

The zone of inhibition diameter of Santan (*I. coccinea*) leaf, Cacao (*T. cacao*) pod husk, and Betel Palm (*A. catechu*) seeds extracts on *S. aureus* is presented in three tables (Tables 1-3). The results presented in the tables are averages of three trials of Kirby-Bauer disk diffusion susceptibility test.

Table 1. Zone of inhibition diameters (in mm) of *S. aureus* after treatment of controls and Santan (*I. coccinea*) leaf extracts (n = 3, results depicted as mean ± SD) and one-way ANOVA.

Sample	Zone of Inhibition Diameter	Interpretation
Positive Control (Penicillin)	28.0 ± 0.00	Very Active
Negative Control (Distilled Water)	6.0 ± 0.00	Inactive
Santan (*I. coccinea*) Leaf Extract 25%[a]	7.3 ± 0.58	Inactive
Santan (*I. coccinea*) Leaf Extract 50%[a]	9.0 ± 1.00	Inactive
Santan (*I. coccinea*) Leaf Extract 75%[a]	10.3 ± 0.58	Partially Active
Santan (*I. coccinea*) Leaf Extract 100%[a]	6.7 ± 1.15	Inactive
p-value	<0.001	Significant

[a] Percentage refers to the concentration of the plant extract.

Table 2. Zone of inhibition diameters (in mm) of *S. aureus* after treatment of controls and Cacao (*T. cacao*) pod husk extracts (n = 3, results depicted as mean ± SD) and one-way ANOVA.

Sample	Zone of Inhibition Diameter	Interpretation
Positive Control (Penicillin)	28.67 ± 0.58	Very Active
Negative Control (Distilled Water)	6.0 ± 0.00	Inactive
Cacao (*T. cacao*) Pod Husk Extract 25%[a]	6.0 ± 0.00	Inactive
Cacao (*T. cacao*) Pod Husk Extract 50%[a]	6.0 ± 0.00	Inactive
Cacao (*T. cacao*) Pod Husk Extract 75%[a]	6.0 ± 0.00	Inactive
Cacao (*T. cacao*) Pod Husk Extract 100%[a]	6.0 ± 0.00	Inactive
p-value	<0.001	Significant

[a] Percentage refers to the concentration of the plant extract.

Table 3. Zone of inhibition diameters (in mm) of *S. aureus* after treatment of controls and Betel Palm (*A. catechu*) seed extracts (n = 3, results depicted as mean ± SD) and one-way ANOVA.

Sample	Zone of Inhibition Diameter	Interpretation
Positive Control (Penicillin)	29.0 ± 1.00	Very Active
Negative Control (Distilled Water)	6.0 ± 0.00	Inactive
Betel Palm (*A. catechu*) Seed Extract 25%[a]	6.0 ± 0.00	Inactive
Betel Palm (*A. catechu*) Seed Extract 50%[a]	6.0 ± 0.00	Inactive
Betel Palm (*A. catechu*) Seed Extract 75%[a]	6.0 ± 0.00	Inactive
Betel Palm (*A. catechu*) Seed Extract 100%[a]	6.0 ± 0.00	Inactive
p-value	<0.001	Significant

[a] Percentage refers to the concentration of the plant extract.

Of the four Santan (*I. coccinea*) leaf extracts used, the extract with 75% concentration demonstrated the highest inhibition on the tested bacteria. All concentrations of Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seed extracts exhibited no inhibition on the tested bacteria. One-Way ANOVA revealed that the differences in the zone of inhibition diameter in all extracts on *S. aureus* were significant ($p<0.01$).

4. Discussion

In this study, the antibacterial activity of Santan (*I. coccinea*) leaf, Cacao (*T. cacao*) pod husk, and Betel Palm (*A. catechu*) seed ethanolic extracts against *S. aureus* was assessed using Kirby-Bauer disk diffusion susceptibility test. It was observed that compared to the negative control (distilled water), all Santan (*I. coccinea*) leaf extract concentrations demonstrated higher antibacterial activity as manifested by their zone of inhibition diameters. However, the Santan (*I. coccinea*) leaf extract 25% and 50% concentrations did not demonstrate antibacterial activity. Nevertheless, the Santan (*I. coccinea*) leaf extract 75% exhibited a partially active antibacterial activity against *S. aureus*. Surprisingly, the Santan (*I. coccinea*) leaf extract 100% did not exhibit antibacterial activity despite of its concentration.

In the previous reports, Santan (*I. coccinea*) leaves demonstrated antimicrobial activity against Gram-positive organisms such as *S. aureus* and *Bacillus subtilis* [41,42]. Phytochemical analysis reported that the leaves contained significant amounts of terpenes, sterols, sapogenins, phenols, flavonoids, and alkaloids which are antibacterial compounds [43]. Furthermore, phytochemical determination and thin layer chromatography bioautography of the leaf extracts showed that their antimicrobial activity was credited to the bioactive compounds the plants possessed. These compounds belong to alkaloid, coumarin, flavonoid, phenolic, and terpenoid groups [43]. In the same study, the minimum inhibitory concentrations (MICs) were assessed and ranged from 0.78 to 3.125 mg/ml. MIC is the lowest concentration of a potential antibacterial material that could suppress the visible growth of microorganisms after incubation. Future studies may exploit the MIC of the ethanolic extract of Santan (*I. coccinea*) against *S. aureus*.

It is noteworthy to report that the Santan extract 100% had the lowest antibacterial activity among the concentrations used. Despite its concentration, this result could be attributed to the viscosity of the extract. As observed, this formulation did not easily penetrate the agar medium because it was less diffusible. Other methods of measuring antibacterial activity appropriate in this case such as broth microdilution may be explored in the future.

On the other hand, the Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seeds extracts exhibited no antibacterial effect against *S. aureus*. Cacao contains a variety of chemicals, including bioactive compounds called flavonoids. Its fruit skin contains notable chemical called phenol. Flavonoids exhibit six times antibacterial activity compared to the commercial drugs. Synthetic derivatives of this compound also showed antimicrobial action against MRSA. The derivatives were 20-to-80-fold more potent than commercial drugs [44]. Phenol is a chemical compound which inhibits the growth of microbial pathogens that cause several plant diseases. Previous report demonstrated that fermented cacao pod husks contain bioactive compounds that inhibit *S. aureus* [16].

Meanwhile, previous studies demonstrated that Betel Palm (*A. catechu*) fruit has antibacterial properties against gram-positive bacteria such as *S. aureus* [13,45,46]. Reports also revealed that it contains bioactive components that evoke an inhibitory effect in microorganisms which could treat many diseases, especially parasitic diseases in the digestive system [45,46]. An analysis on the phytochemical constituents of the ethanolic extract showed that it contains alkaloids, saponins, flavanones, tannins and polyphenols which are suggestive of its antimicrobial effect [47].

While these scholarly works described the potential of Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seeds as antibacterial agents, the present study found no antibacterial activity among all concentrations the mentioned plants extract. A phytochemical analysis on Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seeds may be conducted in the future to validate the claims of the previous reports. Another research may be conducted in the future to validate the results of the present study.

Absence of antibacterial activity does not mean that the bioactive compounds are not present in the plant or the plant has no activity against microorganisms. The absence of antibacterial activity may be attributed to several factors. The present study used ethanol as the solvent and other extraction procedures and solvents were overlooked. The stability of the antibacterial constituents may have fluctuated and degraded before the Kirby-Bauer disk diffusion susceptibility test, thereby decreasing its potency. As observed, the Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seeds extracts were viscous even in the 25% concentration. This property could render the material less diffusible, thus could not easily penetrate the agar medium. To control for this viscosity issue, other methods of measuring antibacterial activity such as broth microdilution may be used in the future. Meanwhile, the maturity of the plant materials may affect the amount of bioactive compounds that can be extracted. Therefore, future studies may also consider this factor to optimize the antibacterial potency of the plant materials.

5. Conclusions

Santan (*I. coccinea*) leaf extract with 75% concentration exhibited the highest antibacterial activity on *S. aureus* among all plant extracts used. However, its antibacterial activity was comparatively lesser than that of Penicillin against *S. aureus*. Cacao (*T. cacao*) pod husk and Betel Palm (*A. catechu*) seeds extracts have no antibacterial activity against *S. Aureus*.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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