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Antibacterial and antifungal assays of the stem extracts of two Philippine lianas, *Bauhinia integrifolia* Roxb. and *Strongylodon paucinervis* Merr.

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Abstract. *Bauhinia integrifolia* Roxb. and *Strongylodon paucinervis* Merr. stem decoctions are used by Manobo tribe in Butuan City, Philippines to treat relapse. The fresh latex of *S. paucinervis* is applied to wounds and its decoction is also used to cure dysentery and diarrhea. This study aimed to assess the antibacterial and antifungal potentials of the stem extracts of these liana species against selected test microorganisms. The aqueous and acetone extracts of the stems were prepared and applied to two Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*), two Gram negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*) and two fungi (*Aspergillus niger* and *Saccharomyces cerevisiae*) using disc diffusion method. Among the test organisms, only the Gram positive bacteria were sensitive to the extracts, with the diameter of zone of inhibition ranging from 10.08±0.30 mm to 20.67±0.88 mm. The minimal bactericidal concentration (MBC) of the acetone extracts ranged from 6.25 to 50 mg mL^{-1}, while the aqueous extracts ranged from 25 to 100 mg mL^{-1}. These results suggest that the tribes can continue using these plants as ethnomedicine to prevent wound infection and treat diarrhrea, and new antibacterial agents can be discovered to control pathogenic bacteria under study.
Key Words: *B. integrifolia*, *S. paucinervis*, *S. aureus*, *B. cereus*, antimicrobial assay, liana.

Rezumat. Decocturile de tulpină de la *Bauhinia integrifolia* Roxb. și *Strongylodon paucinervis* Merr. sunt utilizate de către tribul Manobo din Butuan City, Philippines pentru a trata recidivele. Latexul proaspăt de *S. paucinervis* este aplicat pe răni iar decoctul ei este de asemenea folosit pentru a vindeca dizenteria și diareea. Acest studiu urmărește evaluarea potențialul antibacterian și antifungic al extractelor de tulpină din aceste specii de liane împotriva microorganismelor selectate pentru testare. Extractele apoase și acetonice din tulpini au fost preparate și aplicate la două bacterii Gram pozitive (*Bacillus cereus* și *Staphylococcus aureus*), două bacterii Gram negative (*Escherichia coli* și *Klebsiella pneumonie*) și două micromicete (*Aspergillus niger* și *Saccharomyces cereviciae*) utilizând metoda difuzimetrică pe rondele. Printre organismele testate, numai bacteriile Gram pozitive au fost sensibile la extracte având un diametru al zonei de inhibiție ce a variat de la 10,08 ± 0.30 mm la 20,67 ± 0.88 mm. Concentrația bactericidă minimă (CBM) a extractelor acetonice a variat de la 6.25 până la 50 mg mL^{-1}, în timp ce CBM a extractelor apoase a fost cuprinsă între 25 până la 100 mg mL^{-1}. Aceste rezultate sugerează că triburile pot continua utilizarea acestor plante ca medicină populară pentru a preveni infecţia rănilor şi în tratarea diareei, putând fi descoperiţi noi agenți antibacterieni pentru a controla patogenitatea bacteriilor luate în studiu.
Cuvinte cheie: *B. integrifolia*, *S. paucinervis*, *S. aureus*, *B. cereus*, test antimicrobian, liana.

Introduction. When sulfonamide was produced and tested against bacterial infections in 1930s (Jarrell 2012), history of antibiotics begun and continued to develop in the succeeding years but the term antibiotic was first proposed by S. A. Waksman in 1942 (Parasgandola 1990). Then, in 1940s to 1960s the terms antibiotic and chemotherapeutic drug were differentiated. The antibiotics were referred as natural drugs synthesized by some fungi or bacteria while chemotherapeutic drugs were man-made substances. However, these distinctions were discontinued after the successful chemical synthesis of some antibiotics and development of natural products into drugs (Weatherall 2006). Nowadays, thousands of natural, semi-synthetic and synthetic antibiotics are produced to treat various microbial infections that saved countless lives but, regrettably, some of

these substances are toxic and have bad side effects to the consumers. Faced with the fact that numerous known pathogens are sensitive to certain antibiotics yet many are resistant or developed resistance after subsequent exposure to antibiotics, the search for antimicrobial agents that are safe and with broad spectrum is a never ending quest.

Almost all living organisms produced secondary metabolites with antibiotic properties (Berdy 2005) and plants are among these organisms, which served as alternative sources of inexpensive and safe antimicrobials (Doughari et al 2007). Approximately 20% of the plants found in the world have undergone pharmacological or biological test and their systematic antimicrobial screening became a continuous effort to find new compounds, specifically with the potential to act against multi-drug resistant bacteria (Suffredini et al 2004). Some of the plants recommended by herbalist as indigenous medicines are lianas which are commonly known as woody vines. These plants are numerous and exhibited varied growth forms in lowland tropical forests (Kurzel et al 2006) and were found to play important roles in forest diversity and dynamics (Schnitzer & DeWalt 2006). Lianas were observed to limit the growth, survival and reproduction of trees by competing trees' resources below and above ground especially in the canopy. With these negative impacts to some forest floristic species, many considered these plants as nuisance and discriminately cut and leave it to die. However, other people valued lianas due to their economic, food and medicinal uses. Two of the lianas used by Manobo in Butuan City, Philippines as alternative medicines are *B. integrifolia* and *S. paucinervis*.

This study aimed to screen the aqueous and acetonic stem extracts of these two Philippine lianas for antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsiella pnuemoniae, Saccharomyces cerevisiae* and *Aspergillus niger*. The stem decoction of these liana species are used by Manobo tribe in Butuan City to treat relapse, a condition suffered by most mothers during postpartum period and the latex of *S. paucinervis* is used to treat wounds while its decoction is also used for dysentery and diarrhea due to its astringent and bitter taste properties.

Plant Materials Collection and Preparation. The fresh stems and leaves of *Bauhinia integrifolia* Roxb. (Agpoi, common name and Alibangbang, Manobo name), and *Stongylodon paucinervis* Merr (Yanggud, Manobo name) were collected at 09°00.707' N and 125°39.001' E; and 09°00.705' N and 125°38.979' E, respectively in Sitio Tagubon, Anticala, Butuan City. Fresh samples of these plants were submitted to Biodiversity Research and Training Center for Mindanao (BRTCM), MSU-IIT, Iligan City for species taxonomic identification (Figure 1).

Figure 1. The plant samples being used in this study. (A) *B. integrifolia*, (B) *S. paucinervis*, (1) leaves and (2) stems.

The collected stems were chopped into small pieces and ground into fine powder with a knife and rice or cacao seed grinder after air-drying for two weeks. The powered samples were divided into two parts to obtain crude extracts using water and acetone.

Aqueous Extraction. This process was based on the work of Savithramma et al (2011) with few modifications. Fifty (50) grams of air dried powder was added to 400 mL distilled water and shimmered for two hours after quick boiling. The supernatant was collected using clean double layered cotton cloth, since it cannot penetrate the filter paper, and this procedure was repeated using 200 mL distilled water. The collected supernatant was pooled together and concentrated into 50 mL, which is equivalent to 1 gram of plant material per milliliter through steam bath at less than 50 ºC. It was transferred in the dark bottle, then autoclaved at 121 ºC for 20 minutes and stored at 4 ºC.

Acetone Extraction. Fifty (50) grams of air dried powder was added to 200 mL 70% acetone in an Erlenmeyer flask. The flask was covered with aluminum foil for 6 days. The supernatant was collected using Wattman filter paper and concentrated to 50 mL at less than 50 ºC in a steam bath. The extract was placed in dark bottle and store at 4 ºC.

Test Organisms Preparation. Bacterial cultures of *Bacillus cereus* (BIOTECH 1509), *Staphyloccocus aureus* (BIOTECH 1350), *Escherichia coli* (BIOTECH 1098), and *Klebsiella pnuemoniae* (BIOTECH 10286); and fungal cultures of *Saccharomyces cerevisiae* (BIOTECH 2002) and *Aspergillus niger* (BIOTECH 3080) were purchased from Culture Collection, BIOTECH, University of the Philippines, Los Banos, Laguna. The cultures were maintained in slants at 37 ºC. Prior to the assay, the bacteria were pre-cultured in nutrient broth for 24 hours, while the fungi were taken from slants that were cultured for 48 hours and transferred in potato-glucose broth. The cultures' density was adjusted based on 0.5 McFarland standards ($1.5x10^8$ CFU/mL).

Antibacterial and Antifungal Assays. The methodology on antimicrobial activity of the extracts was adopted from the work of Guevara (2005). Three (3) mL of the extracts were taken from the stocks and evaporated to incipient dryness through steam bath at less than 50°C. A sterile cotton applicator was dipped into the broth medium and the test microorganisms were spread into their respective sterile culture medium (Nutrient Agar for the bacteria and Potato Dextrose Agar for the fungi). The sterile paper discs (6 mm in diameter) impregnated with the extracts, acetone (negative controls), chloramphenicol (commercial antibacteria) and clotrimazole (commercial antifungi) were placed on the test-organism seeded plates in triplicate. The diameter (mm) of the zone of inhibition was taken after 24- and 48-hours incubation at 37°C of bacteria and fungi, respectively. The extracts that exhibited antibacterial activity were subjected to a test that determines their minimal bactericidal concentration (MBC) against the test organisms.

Determination of Minimal Bactericidal Concentration (MBC) of the Extracts. An equivalent to 2 grams of plant extract was diluted with 10 mL sterile distilled water in a 50 mL Erlenmeyer flask. The content was swirled and vortexed for 15 seconds and this was set aside for MBC determination. One (1) mL of this mixture is equivalent to 0.2 g or 200 mg of the plant extract. The chloramphenicol was used as positive control, which was prepared by dissolving 0.10 g with 100 mL sterile distilled water in a volumetric flask to make a 1 mg/mL concentration.

The test organisms were grown separately in 5 mL nutrient broth for 24 hours at 37°C. The cultures were then adjusted to the concentration corresponding to a 0.5 McFarland standard. The 0.1 mL of each adjusted inoculum was diluted further with sterile nutrient broth to a final volume of 20.0 mL. This inoculum was immediately used.

Thirteen (13) pieces of cotton plugged tubes were numbered accordingly. Prior to sterilization, 1.0 mL of nutrient broth was introduced into tubes #2 to #11, while in tube #12 2.0 mL of nutrient broth was introduced. In tubes #1 and #2, 1.0 mL of the plant extract was added. Tube #2 was shaken gently and 1.0 mL was withdrawn, and transferred to tube #3. This process was continued until 1.0 mL has been withdrawn from tube #9 and subsequently added to tube #10. Then, 1.0 mL was withdrawn from tube #10 and this was discarded. After each process, the tube was plugged immediately to prevent contamination. To tubes #1 to #11 and #13, 1.0 mL of bacterial inoculum

previously prepared was added. The tubes were gently vortexed for 15 seconds. After incubating the cultures at 37°C for 24 hours, the tubes were examined for bacterial growth.

The tube with the lowest concentration of the plant extract, that gave no visible growth or turbidity, and the succeeding tubes with visible growth were gently shaken to homogenize. Then, 0.01 mL was withdrawn and spread on the nutrient agar plate using sterile cotton applicator in triplicate. The plates were inverted and incubated at 37°C for 24 hours. The plant extract concentration of the tube producing one colony or no colony at all was reported as the minimal bactericidal concentration.

Statistical Analysis. The collected data on antibacterial and antifungal assays were presented in means with its standard error for three replicates. The means were compared based on the ranged introduced by Guevara (2005).

Results and Discussion. The results of the assessment of the crude extracts of two Philippine liana species against bacteria and fungi are presented in Table 1 and Figure 2. It shows that the commercial antimicrobial agents chloramphenicol and clotrimazole were very potent against the test microorganisms. The antifungal activities of extractant (acetone) and both aqueous and acetone extracts of two plants were negative. The extractant also did not show activity against all bacterial strains while the aqueous and acetone extracts of both plant species showed varying levels of activities against the two Gram positive strains, *B. cereus* and *S. aureus*. Tomas-Barberan et al (1988) and Duraipandiyan et al (2008) observed that the Gram positive bacteria are more sensitive and less resistant to some antibacterial agents than the Gram negative bacteria. This could be due to the differences in their cell wall components, wherein the extracts prevent cell wall formation or alter the cell wall structure of Gram positive bacteria, which resulted to the lysis and eventually death of the cell, or else there might be other means that kill the bacteria since the antibiotic-mediated cell death involved multilayered mechanisms (Kohanski et al 2010).

Table 1

Antimicrobial activity of aqueous and acetone crude extracts of two liana species stem against some microorganisms

	Zone of Inhibition (mm)*					
	Gram (+) Bacteria		Gram (-) Bacteria		Fungi	
Extracts	*B. cereus*	*S. aureus*	*E. coli*	*K.pnuemoniae*	*S. cerevisiae*	*A. niger*
B. integrifolia						
Aqueous	13.33±0.33	14.83±0.58	6.00±0	6.00±0	6.00±0	6.00±0
Acetone	20.67±0.88	18.0±0.58	6.00±0	6.00±0	6.00±0	6.00±0
S. paucinervis						
Aqueous	10.58±0.22	10.08±0.30	6.00±0	6.00±0	6.00±0	6.00±0
Acetone	16.0±1.0	15.67±0.33	6.00±0	6.00±0	6.00±0	6.00±0
Control						
Acetone	6.00±0	6.00±0	6.00±0	6.00±0	6.00±0	6.00±0
Chloramphenicol	41.33±1.86	28.0±0.58	25.33±0.33	23.33±1.20		
Clotrimazole					28.67±1.33	30.67±0.67

Values are presented as mean ± S.E. of triplicate experiment, *Diameter of inhibition zone including diameter of discs 6mm; <10 mm = inactive; 10 – 13 mm = partially active; 14 – 19 mm = active; > 19 mm = very active (Guevara 2005).

Among the acetone extracts, *B. integrifolia* exhibited the highest activities against *B. cereus* (20.67±0.88 mm) and *S. aureus* (18.0±0.58 mm) and *S. paucinervis* acetone extract actively prevented the growth of both bacterial samples (16.0±1.0 and 15.67±0.33 mm, respectively). Similarly, aqueous stem extract of *B. integrifolia* marked the highest zone of inhibition against *S. aureus* (14.83±0.58 mm) and it showed partial activity against *B. cereus* (13.33±0.33 mm). In the same manner, *S. paucinervis* aqueous extract exhibited partial activity against both Gram positive strains (10.58±0.22 and 10.08±0.30, respectively). Forbes et al (2007) mentioned that some strains of *S. aureus* and *B. cereus* are penicillin resistant. They described that *S. aureus* is the normal

flora of the skin and other mucosal surfaces that may cause localized skin infection and deep infections that could spread to other internal organs. Unlike *S. aureus*, *B. cereus* transiently colonized skin or the gastro-intestinal or respiratory tracts and are known as opportunistic among the *Bacillus*. It causes food poisoning that will lead to either diarrheal type, which is characterized by abdominal pain and watery diarrhea, or emetic type, that is manifested by profuse vomiting. Thus, killing of these bacteria is an important combat to save and prolong human lives. This finding would mean that the growth of other Gram positive bacteria could be controlled by the bioactive component(s) possessed by these lianas.

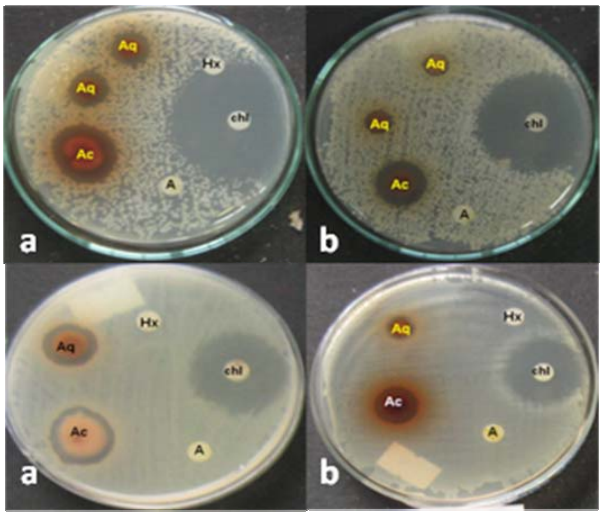

Figure 2. Antibacterial activity of aqueous and acetone extracts of two liana species against *Bacillus cereus* (upper plates) and *Staphylococcus aureus* (lower plates). (a) *B. integrifolia* (b) *S. paucinervis*. A – Acetone (- control) Ac- acetone extract, Aq – aqueous (decoction), Hx- hexane , chl – Chloramphenicol

The minimal bactericidal concentration (MBC) of both aqueous and acetone extracts of the liana species against *B. cereus* and *S. aureus* are presented in Table 2. The MBC of *B. integrifolia* acetone extract against *B. cereus* and *S. aureus* were 6.25 mg mL^{-1} and 12.50 mg mL^{-1}, respectively. Whereas, the MBC of the acetone extract of *S. paucinervis* against the above-mentioned bacterial species recorded 25.00 mg mL^{-1}. With respect to its aqueous extract, it clearly shows that their MBC increased compared to acetone extract (Table 2). This indicates that the activities of the antibacterial metabolites present in the plants were affected by heat.

Table 2

The Minimum Bactericidal Concentration (MBC) of liana stem extracts required against the test organisms

Test Organism	Aqueous Extract	Acetone Extract
	B. integrifolia (mg mL^{-1})	
B. cereus	25.00	6.25
S. aureus	25.00	12.50
	S. paucinervis (mg mL^{-1})	
B. cereus	50.00	25.00
S. aureus	100.00	25.00

However, it is interesting to note that certain antibacterial metabolites were not totally destroyed even if the aqueous extracts were sterilized at 121 $^{\circ}C$ for 15 minutes prior to test.

The results of this study implied that the Manobo tribe could continue using these two liana species as ethnomedicine. Aside from *S. paucinervis*, the fresh latex of *B. integrifolia* can also be used to cure wounds since their extracts (both acetone and decoction) exhibited antibacterial activities against *S. aureus.* The latex of these liana species has astringent to bitter taste properties that are characteristics of tannins (Ashok & Upadhyaya 2012), which are considered as natural antiseptics since these speed up healing of wounds and inflamed mucous membranes (Okwu & Josaiah 2006). Tannins have the ability to form a protective occlusive layer over the exposed tissue to stop bleeding and keep the wound from being infected while it exerts its anti-inflammatory effect (Ashok & Upadhyaya 2012). In addition, the decoction of these plants could be used in washing wounds and treating dysentery or diarrhea caused by the bacteria under study, especially in rural areas where synthetic medicines are unavailable.

Conclusions. The result of this study showed that *B. integrifolia* and *S. paucinervis* have antibacterial properties against the two Gram positive strains, *Bacillus cereus* and *Staphyloccus aureus*. This suggests that the tribes could continue using this as ethnomedicine in preventing wound infection and treating diarrhea caused by these organisms. The other test organisms, *E. coli*, *K. pneumonia*, *A. niger* and *S. cerevisiae*, are resistant to the extracts.

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