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USEFULNESS OF *BIXA ORELLANA* LEAF EXTRACT AGAINST SOME VETERINARY PATHOGENS

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ABSTRACT

Bixa orellana L. is a highly cultivable plant because of its adaptable characteristic and suitability of growth in open location. Its seeds are used for culinary and industrial purposes. Recently, *B. orellana* is shown to possess bioactive substance that can cure common infections. In this investigation, the potential of *B. orellana* as antibacterial plant is examined through its minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) towards *Staphylococcus aureus, Enterococcus faecalis, Salmonella pullorum* and *Pasteurella multocida.* The leaf part of the plant was infused into ethanolic solvent to extract the bioactive substance. Results showed that all test organisms were inhibited at 0.3125 mg/mL and killed at 2.5 mg/mL of the leaf extract. Of the assayed organisms, *Staphylococcus aureus* appeared to be most susceptible having inhibited at 0.078 mg/mL and killed at 0.3125 mg/mL of the extract. *Enterococcus faecalis* appeared less sensitive and killed at 2.5 mg/mL. In terms of action, *B. orellana* crude leaf extract exhibited bactericidal effect against *Staphylococcus aureus*, *Salmonella pullorum* and *Pasteurella multocida* but bacteriostatic against *Enterococcus faecalis*. The promising performance of *Bixa orellana* in this study provides preliminary basis to explore pharmacological development of the plant into a potential drug.

INTRODUCTION

Bixa orellana (Bixaceae), annatto or achuete, is widespread throughout the tropical regions including the Philippines. *B. orellana* is popular for its culinary and industrial uses by producing an organic dye present in the seed coat called "annatto." This dye with red to orange-yellow colors is widely used as colorant for food, textile and cosmetic products. Its subtle and distinctive flavor makes several cuisines. Folkloric practices, however, demonstrate the pharmaceutical uses of *B. orellana* such as anticonvulsant, analgesic, antidiarrheal, diuretic, and antipyretic (Rhadika, *et al*., 2010). Different parts of the plant proved to be therapeutic in several ways. The seeds and root barks were used to treat gonorrhea, the leaves and roots for epilepsy, dysentery, fever, and jaundice (Joshi, 2000). Recently, *B. orellana* has been associated to becoming a good source of antibacterial components against several human pathogens including *Bacillus cereus* (Ahsan *et al.*, 2009), *Bacillus pumilus* (Castello *et al.,* 2010), *Helicobacter pylori* (Cogo *et al.,* 2010), *Klebsiella pneumoniae* and *Salmonella typhi* (Moon and Moon, 2010), *Staphylococcus aureus* and *Escherichia coli* (Mathur *et al*., 2010; Sumathi and Parvathi, 2011). These findings earn the plant a good alternative for synthetic antibiotics which indiscriminate use led to the emergence of multidrug-resistant pathogens.

Drug resistance seriously threatens treatment of infectious diseases and the risk factors associated with infections with multidrug-resistant pathogens are enormous, hence the global health community should delve into efforts to develop natural products with greater marginal safety, available, inexpensive and fewer adverse effects (WHO, 2002).Prior investigations were done on the antibacterial potential of the different plant parts of *B. orellana* using different solvent system through using agar well or disk diffusion method. This study aims to demonstrate potential antibacterial activity of *B. orellana* leaf extract through determination of its minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) towards selected veterinary pathogens.

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MATERIALS AND METHODS

Preparation of *Bixa orellana* Leaf Extract

Fresh leaves of *B. orellana* collected from Brgy. Makinhas, Baybay City, Leyte were thoroughly washed and carefully examined to make certain that no worm or foreign debris contaminated the samples. After washing, 1000g of leaves were air-dried for three days to remove excess moisture (74.5%) and reduce the final weight to 255g. The dried leaves were soaked in ethanol Pancreac® (1:2 w/v) for 48 hours (Fernandez *et al.*, 2009) and strained using Whatman® filter paper. The filtrate, crude Bixa leaf ethanolic extract, was transferred to a beaker and concentrated on rotary vaporator (60°C) and dried at 40°C to obtain the dry extract. A starting dose of 5mg/mL (Metta *et al.*, 2009) crude extract was subjected to nine serial dilutions producing 5mg/mL to 0.02 mg/mL extract concentrations.

Preparation of Test Organisms

Staphylococcus aureus, Enterococcus faecalis, Pasteurella multocida and *Salmonella pullorum* were obtained from the stock cultures of the Microbiology Laboratory of the College of Veterinary Medicine, Visayas State University, Baybay City, Leyte. To ensure that the organisms were pure, an inoculum of each stock culture was re-streaked onto blood agar plates and incubated at 37°C for 24 hours and inside a candle jar with Gaspak® for *Enterococcus faecalis.* From this culture, a colony was selected and streaked onto nutrient agar slant incubated for 24 hours. To prepare the inocula, 2-3 colonies from the nutrient agar slant were suspended in Mueller-Hinton broth and incubated at 37°C until turbidity equaled 0.5 (10^8CFU/mL) McFarland standards following NCCLS (2000) methods.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Mueller-Hinton broth was used to determine MIC of the crude *Bixa* leaf extract in serial dilution technique. One mL of Mueller-Hinton broth was dispensed in each of 10 tubes. One mL of the *Bixa* leaf extract (5mg/mL) was poured onto the first tube and transferred to the tubes accordingly. Finally, 1 mL of the standardized bacterial inoculum was inoculated in all the tubes. The contents of the tubes were mixed thoroughly and incubated at 37°C for 24 hours. The lowest concentration of the Bixa extract with clear tube (without turbidity) is considered the MIC (LSUHSC, 2011; Metta *et al.,* 2009). The tubes with clear content were selected and an inoculum of each was streaked onto nutrient agar. The MBC is the lowest concentration of the clear tube which was streaked onto nutrient agar with no bacterial growth found after 24 hours of cultivation at 37°C.

RESULTS AND DISCUSSION

The extraction the *B. orellana* leaves using ethanol produced a dark green liquid with strong alcoholic odor and watery consistency. Upon concentration, the *B. orellana* crude leaf ethanolic extract yielded a dark green, sticky consistency liquid with sweet odor. Antibacterial activity of the *B. orellana* leaf ethanolic extract through minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) showed that all test organisms were inhibited at 0.3125 mg/mL and killed at 2.5 mg/mL of the extract (Table 1). Among organisms used, *Staphylococcus aureus* was most susceptible having the lowest MIC at 0.078 mg/mL (Figure 1). The MIC of *Salmonella pullorum* was 0.1563 mg/mL and 0.3125 mg/mL for *Enterococcus faecalis* and *Pasteurella multocida*.

Table 1. Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *B. orellana* against test bacteria

Test bacteria	MIC (mg/mL)	MBC (mg/mL)	MIC/MBC ratio
Staphylococcus aureus	0.078	0.3125	4
Enterococcus faecalis	0.3125	2.5	8
Salmonella pullorum	0.156	0.625	4
Pasteurella multocida	0.3125	1.25	4

To determine that inhibited bacteria were no longer viable, all tubes that showed no visible turbidity were inoculated onto nutrient agar. The lowest MBC was exhibited in *Staphylococcus aureus* at 0.3125 mg/mL while the highest MBC at 2.5 mg/mL was demonstrated in *Enterococcus faecalis* (Figure 2). *Salmonella pullorum* and *Pasteurella multocida* had MBC of 0.625 mg/mL and 1.25 mg/mL, respectively.

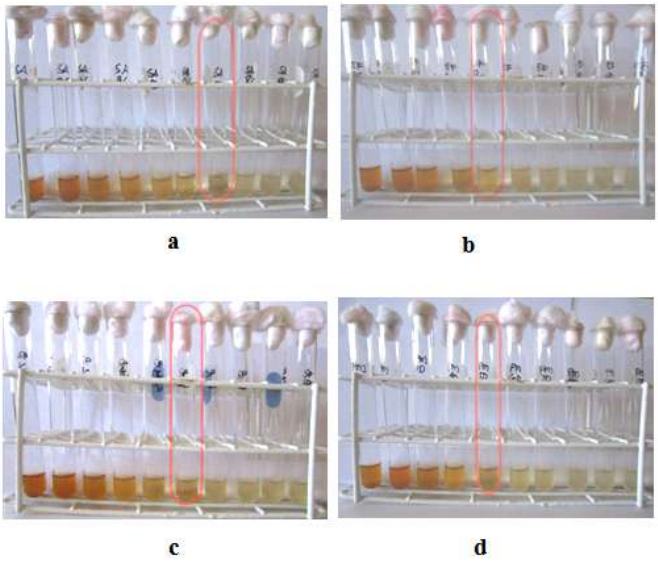

Figure 1. MIC of *Bixa orellana* ethanolic leaf extract against

a) *Staphylococcus aureus* (0.078mg/mL),
b) *Enterococcus faecalis* (0.3125 mg.mL),
c) *Salmonellapullorum* (0.516 mg.mL),
d) *Pasteurella multocida* (0.3125 mg.mL).

Based on the MIC/MBC ratio, there is a fourfold increase in MBC values in *Staphylococcus aureus, Salmonella pullorum* and *Pasteurella multocida* but an eightfold increase in *Enterococcus faecalis.* Various reports indicate that when the MBC value is the same or fourfold higher than its MIC, the bacteria is said to be susceptible and the drug has bactericidal effect. Contrarily, if the MBC is many-fold higher (>4-fold) than its MIC, the drug is bacteriostatic. Along this perspective,

B. orellana crude leaf ethanolic extract is found to be bactericidal against *Staphylococcus aureus, Salmonella pullorum* and *Pasteurella multocida* but bacteriostatic against *Enterococcus faecalis.*

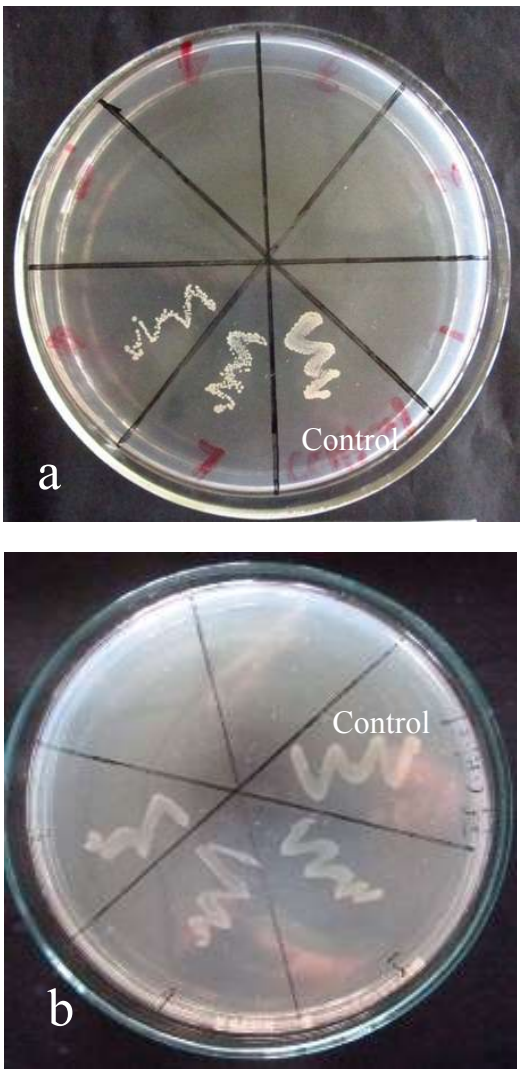

Figure 2. MBC of a) *Staphylococcus aureus* at 0.3125mg/mL and b) *Enterococcus faecalis* at 2.5mg/mL.

Plant extracts are generally more effective against Gram positive than Gram negative bacteria (Suffredini *et al.,* 2006). However, the differential sensitivity of these microorganism to herbal plant extracts may be accounted to their morphological differences and the antimicrobial substances found in the botanical material (Irobi *et al.,* 2010; Silva *et al.,* 2010). The environmental factors where plants are located, the period of plant collection and even the genetic differences in plant species are expected to produce different microbial reactions (Levinson, 2004; Silva *et al.,* 2010). A series of phytochemical screening of *B. orellana* conducted by previous investigators revealed that extraction of bioactive compounds is dependent on the solvent and parts of the plant used. Paiva *et al.* (2010) found that solvents with the same characteristics such as ethanol and methanol having intermediate polarity can extract similar by-products. The use of methanol and ethanol as solvents to both leaves and seeds of *B. orellana* indicated the presence of steroids, flavonoids, tannins, saponins (Tamil, *et al*. 2011; Harborne, 1979; Shilpi *et al.*, 2006), cardiac glycosides, tannin, terpanoids, and anthocyanins (Hajoori, *et al*., 2013; Mathur, *et al*., 2010). Most alkaloids of plant origin are known to act on extra-cellular proteins and inhibit DNA and RNA synthesis (Silva *et al.,* 2010). Flavonoids have the ability to bind with extracellular and soluble proteins and with bacterial cell walls. Lipophilic flavonoids can disrupt bacterial membranes (Clements *et al.*, 2002) which is believed to be the cause of disintegration and aggregation of *Pseudomonas aeruginosa* cells treated with *Bixa orellana* leaf and seed methanolic extract (Latha *et al.,* 2010, Tamil *et al.*, 2011). The antimicrobial activity of saponins, on the other hand, is attributed to its surface-active properties that increases the permeability of bacterial outer membrane imposing cell lysis (Arabski *et al*., 2012). Tannins inhibit bacterial growth and protease activity by damaging the cell wall and cytoplasm causing rapid structural destruction (Andrade *et al*., 2006). Similarly, the mechanism of action of terpenoids is membrane disruption (Jasmine *et al.*, 2011).

In the present study, the activity of *B. orellana* leaf extract against *Staphylococcus aureus* has been consistent with many findings (Fleischer *et al.,* 2003; Metta *et al.,* 2009; Venugopalan and Giridhar, 2012). Other gram-positive bacteria found to be susceptible to *B. orellana* include *Bacillus cereus, Bacillus subtilis* and *Bacillus megaterium* (Hajoori, *et al*., 2013; Mathur, *et al*., 2010) *B. orellana* is also shown to exert notable activity against serious Gram negative pathogens including *Helicobacter pylori, Salmonella typhi, Vibrio cholerae, Pseudomonas aeruginosa, E.coli, Proteus vulgaris,* and *Klebsiella pneumonia* (Cogo *et al.,* 2010; Moon and Moon, 2010; Hajoori *et al*., 2013; Mathur, *et.al*., 2010; Silva *et al.,* 2010). The bacteriostatic activity of *B. orellana* against *Enterococcus faecalis* in this study may be ascribed to the anaerobic condition of the organism during the culture and assay that could have affected its optimum growth compared to other organisms that are grown aerobically (Quinn, 1994). Secondly, *Enterococcus faecalis* is found to develop rather faster resistance to antimicrobials than other organisms because of its intrinsic ability to acquire resistance genes on plasmids (Portenier *et al*., 2003).

Conclusion

Results of the study form a promising basis that *B. orellana* leaf ethanolic extract can be a potential alternative to synthetic antibiotics against veterinary bacterial pathogens. The inhibition of the test organisms entailed that the leaf extract contains broad spectrum phytochemicals. Other investigations showed Bixa as antifungal and anti-parasitic agent (Metta *et al.*, 2009; Moon and Moon, 2010; Rojas *et al.*, 2006; and Tamil *et al.*, 2011). It is implicit therefore that extraction of therapeutic molecules, based on phytochemical screening, be pursued for clinical applications.

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