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Susceptibility of *Streptococcus agalactiae* to aqueous extract of the various parts of acacia (*Samanea saman*)

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Abstract
The study was conducted in order to evaluate the antibacterial potential of acacia leaves, barks and roots aqueous extracts against the bacterium *S. agalactiae* by measuring the zone of inhibition and determining the minimum inhibitory concentration. Extract concentrations that recorded at least 50% survival on the bioassay of Nile tilapia fingerlings were used in the susceptibility test and these concentrations were: leaves = 0, 5 and 10 µg/µl; barks = 0, 5, 10, 20 and 40 µg/µl; and roots = 0 and 5 µg/µl. The zone of inhibition of leaves extract at 5 and 10 µg/µl was statistically significant as compared to 0 µg/µl. For barks extract, concentrations at 10, 20 and 40 µg/µl had significant higher zone of inhibition as compared to 0 µg/µl. Meanwhile, the zone of inhibition of roots extract at 5 µg/µl was significantly higher as compared to 0 µg/µl. The bacterium was still classified as resistant to the various extract concentrations based upon the Clinical and Laboratory Standards Institute (2012). The Minimum inhibitory concentration (MIC) result showed that acacia extracts from leaves, barks and roots were not capable of 100% inhibition of the bacterium. Even though the result was inconsistent, highest percent inhibition was recorded at 10 µg/µl for leaves, 5 and 20 µg/µl for barks and 400 µg/µl for roots.

Keywords: *Streptococcus agalactiae*, nile tilapia, zone of inhibition, Minimum inhibitory concentration

1. Introduction
Tilapia aquaculture in the Philippines is one of the fastest growing industries. In 2014, tilapia production from aquaculture sector has amounted to 259,198.16 MT [1]. Tilapias were initially considered to be more resistant to a number of diseases compared to other cultured species. However, in more recent times, tilapias have been found to be susceptible to both bacterial and parasitic pathogens such as *Streptococcus* sp., *Flavobacterium columnare*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Ichthyophthirius multifiliis*, *Tricodhina* sp. and *Gyrodactylus nilotis* [2]. The creation of a highly stressful environment for the fish due to intensive farming is one of the main reasons for an increased susceptibility to diseases [3, 4, 5].
As early as 1950s, streptococcal infections were already reported in rainbow trout (*Oncorhynchus mykiss*) farms in Japan [6]. The first report of streptococcosis in tilapia was made by Al-Harbi [7]. Common clinical signs of streptococcosis are loss of appetite, exophthalmia, eye hemorrhages, distended abdomen, curvature of the spinal cord, erratic swimming and difficulty in breating [8]. *Streptococcus agalactiae* and *S. iniae* are the major species that affect the production of tilapias in the world [9].
Various approaches have been identified and tested to address problems on bacterial diseases such as sanitary prophylaxis, improvement of management practices, antibiotics and vaccination [10, 2]. Some of these remedies have disadvantages like the non-availability of commercial vaccines in developing countries [10] and development of multiple antibiotic resistant bacteria due to its indiscriminate use [11].
The use of medicinal plant extracts as an alternative to drugs, chemicals and antibiotics in controlling diseases in animals is becoming a current trend. Further, plant extracts are found to enhance the innate immune response of the host at a low concentration and hence its use is very cost effective [12, 13, 14]. Secondary metabolites such as alkaloids, steroids, tannin, phenol, flavonoids and others play a role in the medicinal and antibacterial properties of plants [15].
The main objective of this study was to evaluate the antibacterial potential of acacia (*Semanea saman*) aqueous extract against *S. agalactiae.* Specifically, the study aimed to determine the toxicity of matured acacia leaves, barks and roots aqueous extracts to Nile tilapia fingerlings;

and to compare the antibacterial activity of matured acacia leaves, barks and roots aqueous extracts by measuring the zone of inhibition and determining the minimum inhibitory concentration.

2. Materials and Methods

2.1 Collection and drying of samples

The various parts of acacia that were used in the experiment were collected near the Freshwater Aquaculture Center – Central Luzon State University (FAC-CLSU) fishponds. The matured acacia leaves were sun-dried for 1 hour and other parts such as barks and roots were air-dried for 7 days. In order to attain the desirable moisture content, the plant parts were subjected to 4 hours drying at 70 ˚C using a drying oven.

2.2 Bioassay

Plastic containers of at least 2 L capacity were filled with 1 L of tap water. Ten extract concentrations per plant part were evaluated: 0, 5, 10, 20, 40, 60, 80, 100, 200 and 400 µg/µl. A known weight of plant part was boiled in distilled water for 1 hour to obtain the concentration of stock solution. The mixture was filtered using a clean cloth. The stock solution was diluted at the time of bioassay to obtain the working solution of 1 L. The volume of stock solution that was used in each concentration of working solution was computed using the formula, $C_1V_1 = C_2V_2$, where C_1 = concentration of stock solution, V_1= volume of stock solution, C_2= concentration of working solution and V_2= volume of working solution (Table 1). Each container was stocked with 20 pieces, size 22 pre-conditioned tilapia fingerlings. Mortality was recorded after 24 and 48 hours.

Table 1: Computed volume of stock solution in each concentration of working solution.

Concentration of Stock Solution (µg/µl)	Volume of Stock Solution (mL)	Concentration of Working Solution (µg/µl)	Volume of Working Solution (mL)
500	0	0	1,000
500	10	5	1,000
500	20	10	1,000
500	40	20	1,000
500	80	40	1,000
500	120	60	1,000
500	160	80	1,000
500	200	100	1,000
500	400	200	1,000
500	800	400	1,000

2.3 Estimation of lethal concentration

Lethal concentration of each extract was determined by plotting the extract concentrations against fish mortality after 48 hours exposure. Median lethal concentration (LC50) was estimated by trendline analysis using linear regression in Microsoft Excel. Extract concentrations with at least 50% survival were used in the next phase of the experiment.

2.4 Preparation of filter paper discs

Using a puncher, holes of approximately 7 mm were made from Whatman Filter Paper No. 3. The discs were autoclaved at 15 lbs pressure for 30 minutes.

2.5 Preparation of plant extract stock solution

A known weight of pulverized plant was boiled in distilled water for 1 hour to obtain the concentration of stock solution. The mixture was filtered using Whatman filter paper No. 3. The stock solution was diluted at the time of disc preparation to obtain the working solution of 10 mL. The volume of stock solution that was used in each concentration of working solution was computed using the formula, $C_1V_1 = C_2V_2$ (Table 2).

Table 2: Computed volume of stock solution in each concentration of working solution.

Concentration of Stock Solution (µg/µl)	Volume of Stock Solution (mL)	Concentration of Working Solution (µg/20µl)	Volume of Working Solution (mL)
20	0.00	0	10
20	0.13	5	10
20	0.25	10	10
20	0.50	20	10
20	1.00	40	10
20	1.50	60	10
20	2.00	80	10
20	2.50	100	10
20	5.00	200	10
20	10.00	400	10

A paper disc of 7 mm diameter can absorb 20 µl of solution. Using a mechanical pipette, a fixed volume of 20 µl was loaded on each disc one by one.

2.6 Drying and impregnation of discs

Without covering the petri dishes, the discs were allowed to dry in a clean isolation chamber at 37 °C for 4 hours. The bacterium *S. agalactiae* was isolated from Nile tilapia and was identified using 16S rDNA sequencing. About 2-3 colonies of the 18-24 hours bacterium was suspended in Trypticase Soy Broth (TSB) and was incubated at 37 °C for 1 to 2 hours. The bacterial suspension was adjusted to 0.5 McFarland turbidity standards and 0.2 mL of it was spread in prepared Trypticase Soy Agar (TSA) plate using a sterile cotton swab. After the inoculum has dried, the prepared discs with extracts were placed on the surface of the inoculated

plate using sterile inoculating loop. The discs were positioned such that the minimum center distance was 24 mm and no closer than 10-15 mm from the edge of the Petri dish. The plates were incubated at 37 °C in inverted position and were observed after 18-24 hours of incubation. Using a ruler, the diameter of the zone of inhibition was measured in millimeters. The susceptible, intermediate and resistant categories were assigned on the basis of the critical points recommended by the Clinical and Laboratory Standards Institute.

2.7 Determination of minimum inhibitory concentration (MIC)

One millilitre of extracts (0, 5, 10, 20, 40, 60, 80, 100, 200 and 400 µg/µl) and 9 mL of TSA was be mixed thoroughly in a sterilized plate and was allowed to solidify at room temperature. Around 3-4 colonies of the bacterium were cultured in tube with 3 mL TSB. The tube was incubated at 37°C for 18-24 hours until it achieves the turbidity of 0.5 McFarland standards. The standardized inoculum was diluted in sterile distilled water (1:10) to obtain concentration of 10^6 CFU/mL. From the diluted inoculum, 0.1 mL was spread on the surface of prepared TSA plates. The plates were incubated at 37 °C for 18-24 hours. The MIC was taken as the lowest concentration that inhibits the growth of the bacterium.

2.8 Statistical analysis

Significant differences in the diameter of zone of inhibition were analyzed using One-way Analysis of Variance and Independent Sample T-test. For the comparison of means, Tukey's test was used.

3. Results and Discussion

3.1 Bioassay and estimation of lethal concentration

Bioassay is an analytical method to determine the potency of an extract by its effect on living organisms *in vivo* or *in vitro* [16]. A bioassay experiment can either be qualitative or quantitative, direct or indirect [17]. In this present study, the toxicity of 10 extract concentrations of laves, barks and roots of acacia were evaluated to size 22 Nile tilapia fingerlings. All of the fish died at 40, 200 and 10 µg/µl extract concentration of leaves, barks and roots, respectively. Acacia root extract was considered to be the most toxic followed by leaves and least toxic was extract from barks (Table 3). Acacia extract contained two macrocyclic spermine alkaloids-pitheceolobine-1-2 compounds which were reported to exhibit good cytotoxic activity [18]. Trendline analysis using MS Excel revealed that the median lethal concentration (LC50) of acacia leaves and barks was 10 and 48.61 µg/µl, respectively. LC50 of acacia root extract was not possible to compute using the generated data, thus, the author tried to compute its LC70 which was 7.19 µg/µl. Extract concentrations that recorded at least 50% survival on Nile tilapia fingerlings were used in the susceptibility test and these concentrations were: leaves = 0, 5 and 10 µg/µl; barks = 0, 5, 10, 20 and 40 µg/µl; and roots = 0 and 5 µg/µl.

Table 3: Mortality (%) of size 22 Nile tilapia fingerlings subjected to different extract concentrations of acacia leaves, barks and roots.

Extract concentrations (µg/µl)	Mortality (%)		
	Leaves	Barks	Roots
0	10	0	10
5	15	15	40
10	50	25	100
20	90	25	100
40	100	30	100
60	100	85	100
80	100	90	100
100	100	95	100
200	100	100	100
400	100	100	100

3.2 Zone of inhibition of acacia extract concentrations against *S. agalactiae*

There was significant improvement in the zone of inhibition of acacia extracts against *S. agalactiae* when compared to the control disc or disc without acacia extract. In general, the zone of inhibition increases as the extract concentration increases. The zone of inhibition of leaves extract at 5 and 10 µg/µl was statistically significant compared to 0 µg/µl. For barks extract, concentrations at 10, 20 and 40 µg/µl had significant higher zone of inhibition as compared to 0 µg/µl. Meanwhile, the zone of inhibition of roots extract at 5 µg/µl was significantly higher as compared to 0 µg/µl. No statistical significance was observed when leaves extract was compared to barks and roots extract at 5 µg/µl, and leaves extract when compared to barks extract at 10 µg/µl. This present study has provided general information that acacia extracts from leaves, barks and roots even at low concentration of 5 µg/µl could inhibit the growth of the bacterium *S. agalactiae* (Table 4). Even though there was improvement in the zone of inhibition, the Clinical and Laboratory Standards Institute [19] still classified the bacterium as resistant ($\leq$ 14 mm zone of inhibition) to the various concentrations of extracts used.

Table 4: Zone of inhibition of acacia leaves, barks and roots extract concentrations against *S. agalactiae*.

Extract Concentrations (µg/µl)	Zone of Inhibition (mm)		
	Leaves	Barks	Roots
0	7.00 ± 0.00^{b}	7.00 ± 0.00^{c}	7.00 ± 0.00^{b}
5	8.38 ± 0.52^{a}	7.75 ± 0.71^{bc}	8.00 ± 0.76^{a}
10	8.25 ± 0.46^{a}	8.25 ± 0.46^{ab}	--
20	--	8.63 ± 0.74^{a}	--
40	--	8.75 ± 0.71^{a}	--

Note: Different superscripts were significant at $p<0.05$ level.

In the Philippines, a decoction of the inner bark and fresh leaves are used for diarrhea. The boiled bark is applied as poultice to cure constipation [20]. The roots are used against cancers and/or tumors, tuberculosis and indurations of liver and spleen [21].

The antibacterial properties of *Acacia* plants have been related to antimicrobial compounds such as glycosides, tannins, phenolic compounds, terpenoids, alkaloids, and flavonoids [22]. Specifically for acacia leaves extract, the combinations of chemical compounds (e.g. propanoic acid, ethyl ester, 1-acetoxy-2-propanol, 1,2-ethanediol, diacetate, benzyl alcohol, 1,2,3-propanetriol, 1-acetate, resorcinol, n-pentadecanol,

pentadecanal, octacosanol, neophytadiene, palmitic acid, phytol isomer, supraene, methyl commate b, vitamin E, farnesyl bromide, etc.) might contribute to the effective antibacterial properties of the extract [23]. A literature search on the chemical constituents of acacia bark revealed the presence of two different compounds of alkaloids - $C_8H_{17}ON$ and $C_{17}H_{36}ON_3$ (pith colobine and a saponin samarin). Some other constituents identified in the bark are sucrose, glucose, gallic acid, fatty acids, phytosterol, octacosanoic acid**,** lupeol, α-. spinasterol, α-spinasterone and lupenon [24]. In a study done by Gonzales and Tolentino [25], a 400 g ground acacia bark yielded 2.85 g of alkaloid-rich fraction and percentage yield of 0.71%. On the other hand, Dimaandal [26] stated that acacia leaves gave 0.25% percent alkaloids only. No literature was found on specific phytochemicals found in roots.

The difference in zone of inhibitions obtained using various plant parts was due to differences in the phytochemical profiles of each part. For example, the study of Okoro, Kawo and Arzai [27] found that glycosides, resins, alkaloids and flavonoids were not detected in bark. Flavonoids, alkaloids and steroids were detected in the stem and stem wood, whereas, they were absence in the stem bark. Saponins were not detected in any of the three plant materials [28].

The acacia plant extract bactericidal effect is due to ability to alter the normal cell of the bacteria by causing lysis, loss of rigidity, malformation, and death [23]. Ethyl acetate and methanol leaf extracts of acacia showed inhibition against the rice pathogen *Xanthomonas oryzae* pv. oryzae. At 200 mg/mL ethyl acetate and methanol extract, the highest recorded zone of inhibition was 33.33 and 25.78 mm, respectively [23]. In separate study, leaf extract exhibits antiseptic property to gram-positive organisms such as *Staphylococcus aureus*, *Bacillus subtilis* and *Sarcina lutea* and one gram-negative *Escherichia coli* [29]. Two alkaloid extracts from acacia bark have negative activity against the organisms *E. coli* and *B. cereus*. The test organism *S. aureus* was susceptible to the two alkaloids extracts [29]. Banso [30] found out that *Streptococcus viridians* was susceptible to stem bark extract. Very limited studies were found on the susceptibility of *S. agalactiae* against acacia plant extracts. The only found study available online was made by Arias, Gomez, Cudmani, Vattuone and Isla [31] where they evaluated the antibacterial activity of ethanolic and aqueous extracts of acacia on a number of gram-positive bacteria that include *S. agalactiae*. Results showed that tincture, fluid and decoction preparation of acacia leaves and fluid and decoction preparation of flowers had no inhibitory effect on S. agalactiae. Alcoholature preparation of leaves and flowers, tincture preparation of flowers, and decoction preparation of stems recorded a zone of inhibition of 10-15 mm [31]. The zone of inhibition of acacia plant extracts using solvents such as ethanol, methanol and ethyl acetate was wider as compared to the recorded zone of inhibition in this study using water as solvent.

S. agalactiae isolates from tilapia pond soil in Pampanga, Philippines were found susceptible to antibiotics such as gentamicin, nalidixic acid, chloramphenicol and tetracycline, and intermediate to susceptible to penicillin, ampicillin, amoxicillin and vancomycin [32]. Meanwhile, *S. agalactiae* isolates from pond water in the same collection site were resistant to penicillin and ampicillin at 10 µg dose, amoxicillin at 20 µg dose and vancomycin at 30 µg dose, and susceptible to tetracyline at 30 µg dose and chloramphenicol at 30 µg dose [33].

3.3 Minimum inhibitory concentration of acacia extracts against *S. agalactiae*

The MIC result presented in Table 5 provides general information that acacia extracts from leaves, barks and roots were not capable of 100% inhibition of the bacterium *S. agalactiae*. Even though the result was inconsistent, highest percent inhibition was recorded at 10 µg/µl for leaves, 5 and 20 µg/µl for barks and 400 µg/µl for roots. The inconsistency in the result could be due to uneven spreading of the standardized bacterial inoculum on the surface of agar plate.

The MIC of ethyl acetate and methanol leaf extract to *Xanthomonas oryzae* pv. oryzae was 3.13 mg/mL and 1.56 mg/mL, respectively [23]. In a separate study using methanol leaf extract, *Streptococcus faecalis* was inhibited at MIC of 156 g/mL, *S. aureus* at MIC of 626 g/mL and *Proteus vulgaris* at MIC of 5006g/mL [34]. Meanwhile, *S. pyogenes* which is closely related to *S. agalactiae* was not inhibited by acacia bark extract with concentration as high as 400 mg/mL [27].

Table 5: Inhibition (%) of acacia leaves, barks and roots extract concentrations against *S. agalactiae*.

Extract Concentrations (µg/µl)	Inhibition (%)		
	Leaves	Barks	Roots
0	0	0	0
5	10	40	15
10	35	25	15
20	15	40	10
40	10	15	30
60	15	15	25
80	10	15	20
100	5	15	15
200	5	10	40
400	5	10	50

4. Conclusion

The zone of inhibition of leaves (5 and 10 µg/µl), barks (10, 20 and 40 µg/µl) and roots (5 µg/µl) extract was statistically significant compared to 0 µg/µl. The bacterium was still classified as resistant to the various extract concentrations based upon the Clinical and Laboratory Standards Institute (2012). The MIC result showed that acacia extracts from leaves, barks and roots were not capable of 100% inhibition of the bacterium.

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