Synergistic Antibacterial Activity of *Cassia Fistula* Ethanolic Extract with Antibiotics Amoxicillin and Erythromycin against *Staphylococcus Aureus*

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Manuscript submitted July 12, 2020; accepted September 17, 2020
doi: 10.17706/ijbbb.2020.10.4.170-179

Abstract: *Cassia fistula* or *Golden shower* is an ornamental tree found all over southeast asia and it is known for its use in herbal medicine. This study aims to investigate the potential synergism between *Cassia fistula* ethanolic extract and commonly used antibiotic drugs being amoxicillin, a β-lactam class of antibiotic which is bactericidal; and erythromycin, a macrolide that exhibits bacteriostatic activity against the pathogenic bacterium *Staphylococcus aureus.* Experimental research method with ten individual and combinatorial treatments was utilized. Dried leaves of *Cassia fistula* macerated in 70% ethanol then concentrated using rotary evaporator, working concentrations of amoxicillin and erythromycin, and their combinations were used to inhibit the growth of *Staphylococcus aureus*. Two-fold serial dilution method and assessment of the turbidity of samples using visual spectrophotometer were used to determine the individual and combined treatments' minimum inhibitory concentration (MIC). Fractional inhibitory concentration (FIC) index was used to determine the extent of potentiation of the combinatorial treatments with regards to the original MIC of antibiotic drugs. Findings of the study revealed that combination of amoxicillin and extract yielded a 10-fold increase in activity (from 4.69±2.21 μg/ml MIC of drug alone to 0.44±0.21 μg/ml MIC in combination with the extract), much higher than that of Erythromycin and extract combination which yielded a 5-fold increase in activity (from 25.00±0.00 μg/ml MIC amoxicillin of drug alone to 4.69±2.21 μg/ml MIC in combination with extract). Both drug–extract combinations showed synergism as defined by their individual FIC (≤ 0.5).

Key words: Amoxicillin, *Cassia fistula*, erythromycin, antibacterial synergism.

1. Introduction

The advent of antibiotics, since its discovery less than a century ago has shaped the world of modern medicine, pharmacology, and microbiology and saved the lives of millions of people until this day. We have gone a long way from treating wounds and fevers with leaves, flowers, and fruits to mass producing synthetic antibiotics to be used to combat bacteria. A plethora of researches has been done to investigate antibacterial properties of plant-based products, but a modern angle of ethnopharmacology turns its eye towards combinatorial treatments between plant-based products and synthetic drugs with the hopes of creating a more powerful antibacterial agent or to reverse the drug immunity of evolving bacteria [1], which

is arguably one of the most challenging problems being faced today.

An abundance of research on plant extract antibacterial potential has been seen through the years, but it seems that none of these plant derived extracts were exploited for clinical or pharmaceutical use. It was observed that plant compounds generally yield Minimum Inhibitory Concentration (MIC) values of <1000 µg/ml, which were of no relevance or significant in a clinical perspective [2]. Phytochemicals found in plant extracts were postulated to act as pump inhibitors that facilitate better penetration of antimicrobial compounds [3], therefore having a higher rate of antibiotic agent delivery inside a pathogenic cell, thus, combinatorial treatments with plant extracts and antibiotic drugs are being studied. A concept of synergism in pharmacology pertains to potentiation of a certain agent with another agent that produces effects greater than the sum of individual agents, and it shows on studying Multi-drug Resistant Bacteria that synergism between antibiotic drugs and plant extracts were found better in protein synthesis and cell wall biosynthesis inhibitor antibiotics [4], hence this research tackled both types of antibiotic drugs.

Cassia fistula, otherwise known as Golden Shower Tree, known for its beautiful yellow flowers and long thin fruits are widely distributed to Southeast Asia including the Philippines, India, and Thailand. It has been used not just a decorative plant but also to dress wounds and alleviate diarrhea. Phytochemical studies, particularly its leaves have proven to contain a wide variety of bioactive compounds such as tannins, flavonoids, saponins, triterpenoids, among others. Wound healing property of *Cassia fistula* has been attributed to its established antibacterial activity, particularly against *Staphylococcus aureus* [5], one of the most common and infectious bacteria that causes many diseases from a simple pimple to life threatening pneumonia. The antibiotic drugs Amoxicillin and Erythromycin are both effective and medically prescribed against *Staphylococcus aureus*, although both drugs work, they differ in drug classification and mode of action. Amoxicillin is a β-lactam that inhibits cross-linkage of the peptidoglycan cell wall of bacteria to lyse the cell (Bactericidal) [6], while Erythromycin is classified as a Macrolide that acts on the 50S ribosome unit of the bacterial cell and inhibits its protein translation, rendering the cell unable to multiply further (Bacteriostatic) [7]. Hence this research aims to identify which among the two antibiotic drugs with different modes of action would show synergism with a plant extract and which tends to have a greater increase in antibacterial activity.

2. Materials and Methods

2.1. Materials

The preparation of the leaf extract of *Cassia fistula* included the following materials: Dried *Cassia fistula* leaves, 70% ethanol solution, electric blender, storage bottles, beaker, cheesecloth, and rotary evaporator.

In the inoculation and culturing of bacteria and media preparation the materials needed are: Petri dishes, weighing scale, agars and broths, test tubes, alcohol lamp, inoculating loops and needles, stirring rod, hot plate, autoclave, and incubator.

Preparation of antibiotic solutions included: Antibiotic drugs in powder form, weighing scale, beaker, stirring rod, centrifugal tubes, distilled water, and micropipette. Assessment of the turbidity of the samples required a visual spectrophotometer.

2.2. Isolation of Pathogen

Bacterial strain of *Staphylococcus aureus* (ATCC 29213) was obtained from the university of the philippines, manila in a slant agar tube placed inside a sealed biohazard ziploc bag and was stored inside a bacterial refrigerator until used.

The bacterium was inoculated into five sterile petri dishes with Mannitol Salt Agar using multiple interrupted streak technique and incubated for 24 hours under 37 °C [8]. The culture was then used to

obtain a pure colony using a sterilized inoculating needle and placed into a sterilized test tube containing normal saline solution (NSS). Turbidity of the test tube was consistent to 0.5 McFarland Solution [9] to obtain the standard bacterial density of $5x10^5$ colony forming units (CFU) to ensure equal inoculation of the pathogen later in the testing (Fig. 1c).

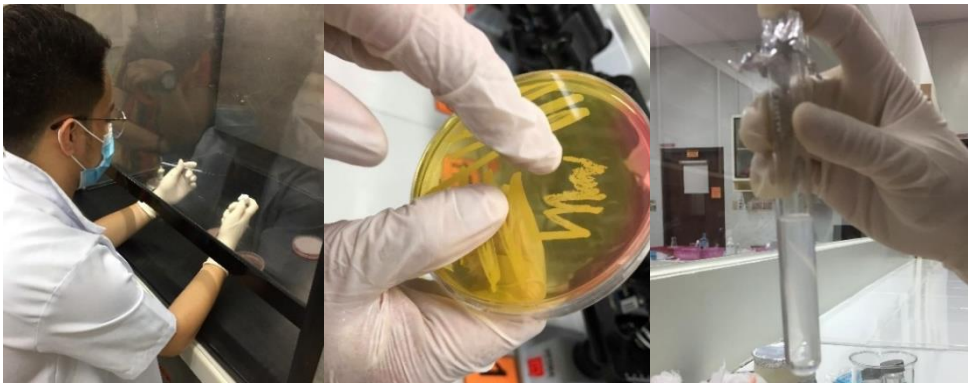

Fig. 1. a). Isolation of *S. aureus*; b). Pure colony identification; c). Bacterial suspension in NSS

2.3. Preparation of Extract

Fresh and healthy leaves of *Cassia fistula* were collected from Bohol, Philippines then transported to Manila. The leaves were authenticated by the Herbarium of the Institute of Biology of the University of the Philippines, Diliman. Five kilograms of the leaves were washed thoroughly and then air-dried [10] to remove excess moisture. The leaves were then powdered using a mechanical grinder for homogenization. A weight of 250 g of the powdered leaves was macerated in 1 liter of 70% ethanol for 3 days with constant agitation (Fig. 2a).

The macerated leaves were then filtered using cheesecloth which yielded 500 ml of dark green color liquid. The liquid solution was then concentrated using a Rotary Evaporator which resulted to a 150 ml strong scented dark green liquid (Fig. 2b) and was stored in an amber reagent bottle and refrigerated until used.

Fig. 2. a). Homogenization of dried *C. fistula* leaves; b). Maceration in 70% Ethanol

2.4. Preliminary Phytochemical Analysis of *C. fistula* Extract

The Phytochemical constituents of different Extraction solvents of *C. fistula* leaves, namely petroleum ether, chloroform, ethanol, methanol and water proved an ample amount of Phytochemicals such as alkaloids, flavonoids, carbohydrates, glycosides, protein and amino acids, saponins, and triterpenoids revealed the presence of most of constituents in polar extracts (ethanol, methanol, and aqueous) compared

with nonpolar extracts (petroleum ether and chloroform) [11].

2.5. Preparation of Antibiotic Stock and Working Solution

The antibiotic drugs were obtained in their standard powder form directly from the manufacturing company which also provided the drug's potency in µg/mg, which will be used in determining the drug's weight to be dissolved in order to obtain an equal potency between the two antibiotic drugs [12]. The Amoxicillin (from Drugmaker's Laboratories, Inc.) had a potency of 574 µg/mg, whereas Erythromycin (from Steinbach Products, Inc.) had a potency of 694 µg/mg. The stock solutions of each antibiotic drugs were prepared by dissolving known weights in 50 ml distilled water and equalized into concentrations of 1,000 µg/ml by factoring in the potencies.

The stock solutions of each antibiotic drugs were prepared using the formula:

$$Weight\ (mg) = \frac{Volume\ (ml) x\ Concentration\ (\mu g/ml)}{Potency\ (\mu g/mg)}$$

Therefore, a weight of 87.11 mg of Amoxicillin and 72.05 mg of Erythromycin were dissolved in 50 ml of distilled water. The stock solutions were further diluted into working concentrations to be used in the Two-fold Serial Macrodilution testing to determine the MIC [13].

The working concentration of each antibiotic drugs were prepared using the formula:

$$Concentration^1\ x\ Volume^1 = Concentration^2\ x\ Volume^2$$

Therefore,

$$Volume^1 = \frac{(100\ \mu g)\ (50\ ml)}{1{,}000\ \mu g}$$

A total of 5 ml of each of the stock solutions were added to 45 ml of distilled water in order to make a 50 ml working concentration solution. The solution was then filtered using filter syringes and placed in centrifugal tubes.

2.6. In vitro Evaluation

2.6.1 Two-fold serial broth macrodilution

The goal of this test is to determine the Minimum Inhibitory Concentration of the extract, antibiotic drugs, and their combinations by culturing the bacterium in decreasing concentrations of the agents [14]. For each agent to be tested, 2 ml Mueller-Hinton Broth was transferred to 11 test tubes, 10 of them will be used in the dilution test representing the samples for the decreasing concentrations of agents and 1 for the negative control (Fig. 3). Prior to autoclaving, the test tubes were plugged with cotton that is wrapped in gauze to inhibit any leakages and prevent contamination.

For the individual tests, the samples were then treated with decreasing concentrations of each of the antibiotic drugs starting from 100 µg/ml until it reaches the 10^{th} test tube in 0.196 µg/ml. The same procedure was done in the *Cassia fistula* extract starting with 1,000 mg/ml until reaching the 10^{th} test tube in 1.95 mg/ml. The concentrations used in the extract was purposely done in a higher concentration in order to attain a broader range of concentrations tested, since earlier trials of this treatment with lower concentrations did not yield any inhibition. The samples were then aliquoted 200 µg/ml of the bacterial suspension with $5x10^5$ bacterial density apart from the negative control and incubated for 24 hours at 37 °C. Combination treatments will also follow the same procedure for synergism testing. Treatments for extract, antibiotic drugs, and combinations are done in triplicates.

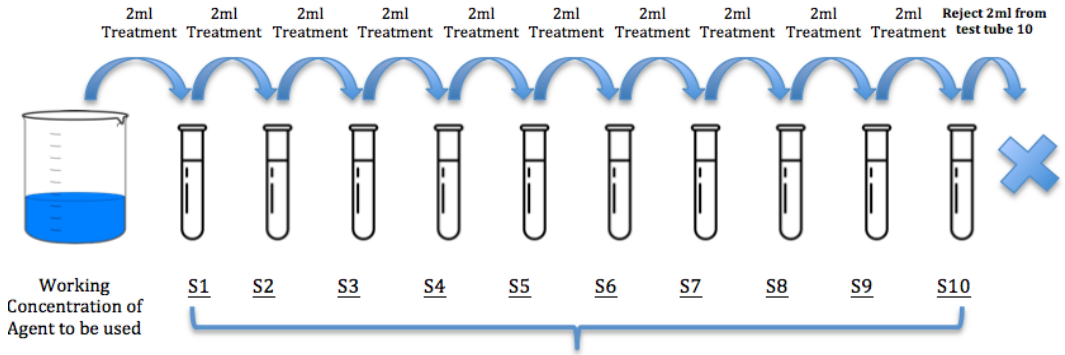

Fig. 3. Design of the two-fold serial macrodilution method.

2.6.2 Spectrophotometric study

Cell viability of the samples is determined by the turbidity of the solution after incubation. Having a highly turbid sample indicates bacterial growth, hence, the turbidity of the negative control (the sample that did not have any bacterial inoculum) is the standard for identifying which sample had no bacterial growth in it. Thus, the sample having the least concentration that matches the turbidity than that of the negative control will be determined as the MIC [15].

In order to have a quantifiable measurement of the turbidity of the samples, a Single - Cell Visual Spectrophotometer (Labomed, Inc.) was used (Fig. 4). A clean test tube with distilled water and an optical density of 600 nanometers [16] were used to calibrate the machine, setting its absorbance tare to 0.00 value. All samples of the agents underwent spectrophotometric study to find the MICs of the agents.

Fig. 4. a). Calibration of spectrophotometer; b). Calibrated spectrophotometer without any samples inside.

2.6.3 Test for synergism

With the use of identifying the Spectrophotometric absorbance in OD_{600nm}, it was made possible to have an analysis on which among the samples were consistent to the absorbance of the negative control, establishing the individual MIC of the extract and antibiotic drugs.

There are only a handful or tests available for the analysis of synergism between a natural product and a synthetic drug. The synergism testing was carried out by combining the MIC of the extract to the MIC of each of the antibiotic drugs in equal ratio to produce a combined solution [17], [18] treatment. The same method of two-fold serial dilution and spectrophotometric study that was used in the individual MIC of the agents will be used in the combination treatments (Fig. 5).

Fig. 5. Serial macrodilution of combination treatments.

The data that will be gathered from both the individual and synergism test MIC will be used to compute for the Fractional Inhibitory Concentration (FIC) Index in line with the checkerboard method in determining the degree of synergism of the agents [19].

3. Results and Discussion

3.1. Minimum Inhibitory Concentration of Individual and Synergism Tests

The Minimum Inhibitory Concentration results of the extract, antibiotic drugs, and combination treatments were expressed as Mean±SD or standard deviation in interpreting the average of their triplicates. The absorbance of the individual tests was compared to the absorbance of the negative control (25.00±0.00 µg/ml). It is noteworthy that the MIC value of the plant extract showed a significantly larger concentration needed to inhibit bacterial growth. This is consistent to earlier claim that plant-based extracts are not medically used as an antibiotic because it's far less potent than the usual antibiotics. Amoxicillin yielded an MIC of 4.69±2.21 µg/ml and Erythromycin 25.00±0.00 µg/ml. The efficacy of the antibiotic drugs somehow dwarfs than that of the extract (Table 1), being that the Amoxicillin was more than 53,000 times more effective and Erythromycin 10,000 times. It is also expected that Erythromycin had a smaller MIC value because unlike Amoxicillin which is a bactericidal antibiotic, it can only be bactericidal (lyse a pathogen) at a higher concentration, its main function is to only inhibit bacterial replication by competitive inhibition of bacterial protein synthesis.

Table 1. Minimum Inhibitory Concentration of the Individual Test and Synergism Test

Treatments	Minimum Inhibitory Concentration (MIC)	Spectrophotometric Absorbance in OD_{600nm}
Individual Test:		
C. fistula Extract	250.00±0.00 mg/ml	0.051±0.0014
Amoxicillin	4.69±2.21 µg/ml	0.056±0.0057
Erythromycin	25.00±0.00 µg/ml	0.059±0.0028
Synergism Test:		
C. fistula Extract + Amoxicillin	0.44±0.21 µg/ml	0.052±0.0014
C. fistula Extract + Erythromycin	4.69±2.21 µg/ml	0.052±0.0000

The ultimate goal of this study is to find out whether a plant-based product can potentiate a common antibiotic. That was made possible by the synergism testing upon the combination of individual MICs of the agents. The combination of *C. fistula* extract and Amoxicillin has produced an astounding MIC value of 0.44±0.21 µg/ml and the combination of *C. fistula* extract and Erythromycin 4.69±2.21 µg/ml.

To have a quantifiable measurement on the extent of potentiation of the combination treatments, the Fractional Inhibitory Concentration (FIC) index was measured.

The FIC is derived from:

$$FIC\ of\ Drug\ A: \frac{MIC\ of\ drug\ A\ when\ tested\ in\ combination\ with\ drug\ B}{MIC\ of\ drug\ A\ alone}$$

$$FIC\ of\ Drug\ B: \frac{MIC\ of\ drug\ B\ when\ tested\ in\ combination\ with\ drug\ A}{MIC\ of\ drug\ B\ alone}$$

$$\Sigma FIC = FIC^A + FIC^B$$

This is a checkerboard analysis used in Microbiology to classify whether the combination is Additive/Indifference as defined as ΣFIC of 0.5< to ≤4, Antagonistic as ΣFIC of >4, or Synergistic as ΣFIC of ≤0.5. In the equation, the Drug A will be represented individually by the antibiotic drugs, and the drug B will be represented by the extract. An FIC for both the combinations of extract + Amoxicillin and extract + Erythromycin was computed.

From the data obtained and presented in Table 2, it showed that both antibiotic drugs in combination with the extract a ΣFIC of ≤0.5, thus the combination treatments of antibiotic drugs and *C. fistula* extract was definitively synergistic against *Staphylococcus aureus*. The synergistic effect of the agents was well within the standard of exemptional potentiation. It was reflected that Amoxicillin, when combined to *C. fistula* extract would increase its efficacy 10 times, wherein Erythromycin yielded an increase of 5 times its efficacy. Both MIC of antibiotic drugs were raised by the extract drastically. the results revealed that the extract can potentiate Amoxicillin better than Erythromycin.

Table 2. Fractional Inhibitory Concentration Index

Combination treatments	FIC^A	FIC^B	ΣFIC	Effect	Fold increase in efficacy
C. fistula Extract + Amoxicillin	0.09	0.0018	0.0918	Synergistic	10-fold
C. fistula Extract + Erythromycin	0.18	0.019	0.199	Synergistic	5-fold

* FICA: Fractional Inhibitory Concentration of Amoxicillin/Erythromycin.
* FICB: Fractional Inhibitory Concentration of *C. fistula* extract.

4. Conclusion and Recommendation

Evidence of the ability of *C. fistula* ethanolic extract to improve the efficacy of antibiotic drugs Amoxicillin and Erythromycin has been proven by this study, opening a possibility to conduct further studies with other antibiotic drugs of different classes and other pathogenic bacteria. This should serve as a baseline knowledge in natural product-antibiotic combinatorial treatment

The extract potentiated, although both yielded a synergistic response, a bacterial wall synthesis inhibitor Amoxicillin more than the protein synthesis inhibitor Erythromycin. This study suggests the possibility of concurrent use of these antimicrobial drugs and extracts in combination in treating infections caused by S. aureus strains or at least the concomitant administration of these plants and antimicrobial drugs may not impair the antimicrobial activity of these antibiotics.

Further studies on the capability of drug-extract synergism to reverse the resistance of different multidrug-resistant bacteria are already being made. Testing the synergism of these agents against Methicillin-Resistant *Staphylococcus aureus* (MRSA) is indeed the next step for this research. Other studies of combinations of different classes of antimicrobials are also imperative. Clinical controlled studies are needed to define the real efficacy and possible toxic effects of this combinations *in vivo*.

5. Limitations of the Study

The research only investigated the combinatorial treatments on positive (+) gram bacteria as represented by *Staphylococcus aureus.* It also did not include other antibiotic class that renders a different mechanism of action apart from Macrolide and β-lactam antibiotic mechanisms. The Macrodilution method used offered a limited capacity of tests since test-tubes occupy a relatively larger amount of space in contrast to its smaller and more efficient counterpart in Microdilution – the microplate.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgment

The authors acknowledge the invaluable contribution and influence of Dr. Alonzo Gabriel, a Filipino Scientist from the University of the Philippines - Diliman who have recently passed, to this research. The authors also would like to express their gratitude on the College of Arts and Sciences and the Science Laboratories of San Beda University – Manila for their continuous support and inspiration.

Author Contributions

With Mr. Jansel Rañin as the head/corresponding author, he is the main proponent of the study and handled the certifications and procurement of materials. Ms. Lailanie Evangelista, as his research adviser, worked with him in the methodologies (agent preparation, antibacterial studies, and spectrophotometry) and the theoretical framework of the study. Dr. Liwayway Acero, as the thesis professor, was primarily concerned on the title approval, paper content, and statistical methods.

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