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Antibacterial Activity of Cacao (Theobroma Cacao Linn.) Pulp Crude Extract Against Selected Bacterial Isolates

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Abstract - Cacao (Theobroma cacao Linn.), a plant with great perennial leaves and yellow or reddish flowers is reported to be antiseptic, diuretic, ecbolic (promotes labor by stimulating uterine contractions), emmenagogue (promotes or stimulates menstrual flow), and parasiticide, Cacao is a folk remedy for alopecia, burns, cough, dry lips, eyes, fever, listlessness, malaria, nephrosis, parturition, pregnancy, rheumatism, snakebite, and wounds. With its wide range of medicinal values, its activity against selected bacterial isolates will be determined in order to find alternative substances that will prevent the widespread of bacterial infection. This study used the experimental research method to determine the antibacterial activity of cacao (Theobroma cacao Linn.) pulp crude extract against selected bacterial isolates including

Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 using the paper disc diffusion method. The Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and the biological stability of cacao against the test microorganisms were also determined using the tube dilution method. Stability tests were also performed such as the detemination of the effects of pH and temperature on the activity of cacao against bacterial isolates.

Keywords - Cacao, Theobroma cacao, Bacterial Isolates

INTRODUCTION

Microorganisms are living organisms that are too small to be seen by the naked eye. They are commonly known as "germs". They are said to be ubiquitous meaning they are virtually everywhere. They are usually microscopic and are called microbes. Most of them are unicellular. They can be divided into those that are truly cellular such as bacteria, archaeans, algae, protozoa and fungi; and those that are acellular such as viruses, viroids and prions. They play significant roles in our lives. They may be pathogenic, non-pathogenic or a normal flora, or opportunistic pathogens of human body (Engelkirk and Burton, 2007).

Microorganisms are responsible for almost all of the common infectious diseases. Interestingly enough, many disorders formerly thought to be caused by other factors, like stress, are now known to be caused by bacteria. They can cause diseases such as skin diseases, UTI, gangrene, ulceration, wounds, burns, respiratory infections, sepsis, bacteremia, syphilis, herpes, gastrointestinal infection, diarrhea, enteritis, pneumonia, tuberculosis and many more. And because of those pathogenic activities of microorganisms, science is finding way on how to eliminate them and that made the discovery of antimicrobial cure.

Organisms in each domain share properties of their cells that distinguish them from members of different domains because genes

were transferred between domains billions of years ago and the three domains may have a common ancestor. The three domains are the Bacteria, Archea and Eucarya (Nester, et.al., 2007).

Bacteria are all single celled organism with specific shapes, most commonly cylindrical (rod-shaped), spherical (round) or spiral. Most of then have rigid cell walls, which are responsible for the shape of the organism. The walls contain an unusual chemical compound called peptidoglycan, which is not found in other domains. They multiply by binary fission in which one cell divides into two cells, each generally identical to the original cell. They are categorized in groups, Gram-positive cocci, Gram-positive bacilli, Gram-negative cocci, Gram-negative bacilli, Enterobacteriaceae, and Anaerobes (Nester et al., 2007).

Staphylococcus aureus is a catalase-positive, gram positive coccus, usually arranged in clusters. In the laboratory, S. aureus can be differentiated from other Staphylococcus species of human origin by using the coagulase test. S. aureus is coagulase-positive meaning that it produces the enzyme coagulase, whereas other staphylococci are coagulase-negative. S. aureus is a facultative anaerobe and opportunistic pathogen that is often found in low numbers as indigenous microflora of the skin. Infections caused by the S. aureus are often referred to as staph infections. It is a major cause of the skin, soft tissue, respiratory, bone, joint, endovascular and wound infections. Most pimples, boils, carbuncles and styes involve S. aureus. It is less common cause of pneumonia and urinary tract infections. S. aureus is one of the four most common causes of nasocomial infections, often causing post-surgical wound infections. Strains of S. aureus produce a variety of exotoxins, including cytotoxins, exfoliative toxins, and leukocidin. Some strains produce toxic shock syndrome -1 (TSS-1) toxin, the cause of toxic shock syndrome. Those strains that produce enterotoxin) causes staphylococcal food poisoning, one of the most common type of food poisoning. Strains of S. aureus produce a variety of exoenzymes including protease, lipase, and hyaluronidase that destroy tissues, coagulase that can cause clot formation and staphylokinase that dissolves clots (Engelkirk and Burton, 2007).

During the late 1950s and early 1960s, S. aureus caused considerable morbidity and mortality as a nosocomial, or hospital-acquired,

pathogen. Since then, penicillinase-resistant, semisynthetic penicillins have proved to be successful antimicrobial agents in the treatment of staphylococcal infections. Unfortunately, methicillin-resistant S. aureus (MRSA) strains have recently emerged as a major nosocomial problem (Prescott et.al., 2005).

Escherichia coli is one of many species of bacteria living in the lower intestines of mammals, known as gut flora. When located in the large intestine, it assists with waste processing, vitamin K production, and food absorption. Discovered in 1885 by Theodor Escherich, a German pediatrician and bacteriologist, E. coli are abundant. The number of individual E. coli bacteria in the feces that a human defecates in one day averages between 100 billion and 10 trillion. However, the bacteria are not confined to this environment, and specimens have also been located, for example, on the edge of hot springs. E. coli generally ferments lactose. It is an almost universal member of the normal intestinal flora of humans and a number of other animals. E. coli are unable to sporulate. As a result of their adaptation to mammalian intestines, E. coli grows best in vivo or at the higher temperatures characteristic of such an environment, rather than the cooler temperatures found in soil and other environments (Nester, et.al, 2007).

E. coli can generally cause several intestinal and extra-intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and Gram-negative pneumonia (Wikipedia [Online], 2007).

The E. coli strain O157:H7 is one of hundreds of strains of the bacterium that causes illness in humans. It causes bloody bacteria that sometimes develop into hemolytic uremia syndrome which is life threatening. E. coli 0157:H7 has caused several large food poisoning outbreaks. Prevention then involves more thorough cooking, so that enough heat reaches the center to kill all the organisms (Nester, et.al, 2007).

Sanitary precautions including careful hand washing, pasteurization of drinks, through cooking of meats, replacement of fluid loss, antibiotics and bismuth compounds were used for treatment.

Pseudomonas aeruginosa is the type species of the genus Pseudomonas. It is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. It is a common environmental bacteria that as

a group can degrade a wide variety of compounds. It is a ubiquitous microorganism in the hospital environment, existing almost anywhere there is moisture, including medical equipment and disinfectant solutions and soaps. It is only rarely found as part of the normal flora of healthy people. It may produce serious infections in patients with burns, traumatic, and operative wounds. It also produces a slime polysaccharide, an endotoxin, and proteases that inactivate components of complement, thereby inhibiting to some degree opsonization and the inflammatory responses and perhaps contributing to its invasiveness (Henry, 2007). An opportunistic human pathogen, P. aeruginosa is also an opportunistic pathogen of plants.

P. aeruginosa secretes a variety of pigments, including pyocyanin (blue-green), fluorescein (yellow-green and fluorescent, now also known as pyoverdin), and pyorubin (red-brown). King, Ward, and Raney developed Pseudomonas Agar P (King A media) for enhancing pyocyanin and pyorubin production and Pseudomonas Agar F (King B media) for enhancing fluorescein production.

Recently, a form of tobramycin that can be administered through inhalation rather than injection was developed, making lung infections in cystic fibrosis patients caused by P. aeruginosa safer and more effective (Nester, et.al, 2007).

FRAMEWORK

The history of antimicrobials begins with the observations of Pasteur and Joubert, who discovered that one type of bacteria could prevent the growth of another. They did not know at that time that the reason one bacteria failed to grow was that the other bacteria were producing an antibiotic. Technically, antibiotics are only those substances that are produced by one microorganism that kill, or prevent the growth, of another microorganism. In today's common usage, the term antibiotic is used to refer to almost any drug that cures a bacterial infection. Antimicrobials include not just antibiotics, but synthetically formed compounds as well (Nester, et.al., 2007).

Antimicrobial drugs are drugs designed to kill or inhibit the growth of microorganisms. Those that inhibit bacterial growth are called bacteriostatic. These drugs depend on the normal host defenses

to kill or eliminate the pathogen after its growth has inhibited. Drugs that kill bacteria are bactericidal. These drugs are particularly useful in situations in which the normal host defenses cannot be relied on to remove or destroy pathogens. A given drug can be bactericidal in one situation yet can be bacteriostatic in another, depending on the concentration of the drug and growth stage of the microorganism (Nester, et.al, 2007).

Interestingly enough, many disorders formerly thought to be caused by other factors, like stress, are now known to be caused by bacteria. An antimicrobial agent must inhibit or destroy the pathogen without damaging the host. To accomplish this, the agent must target a metabolic process or structure possessed by the pathogen but not possessed by the host.

However, the future effectiveness of antimicrobial therapy is somewhat in doubt. Microorganisms, especially bacteria, are becoming resistant to more and more antimicrobial agents. Just as humans are assembling a vast array of antimicrobial drugs, microorganisms have their own genetic toolbox of mechanisms to avoid their effects. In some cases, certain types of bacteria are inherently resistant to the effects of particular drug (Nester, et. al, 2007). Bacteria found in hospitals appear to be especially resilient, and are causing increasing difficulty for the sickest patients–those in the hospital. Currently, bacterial resistance is combated by the discovery of new drugs. However, microorganisms are becoming resistant more quickly than new drugs are being found, thus, future research in antimicrobial therapy may focus on finding how to overcome resistance to antimicrobials, or how to treat infections with alternative means.

Cacao (Theobroma cacao Linn.) is classified in the family of the Sterculiaceae (Gears [Online], 2007). The local names of the tree are Cacao both in Spanish and Tagalog and Cocoa in English. It is widely scattered in cultivation at low and medium altitudes but is nowhere spontaneous in the Philippines. It was introduced to the country for cultivation from Mexico during the Spanish occupation (Stuartxchange [Online], 2009).

The cacao is a small tree growing from 3 to 5 meters in height. The leaves are oblong-obviate to oblong, and 15 to 40 centimeters in length, with pointed tip and rounded base. The flowers are solitary or fascicled

on the trunk and branches, yellowish or nearly white, and about 1 centimeter in diameter. The fruit is oblong, 10 to 15 centimeters long, prominently wrinkled, yellow or purplish. The seeds are numerous and embedded in whitish pulp.

There are three main varieties of the Theobroma cacao: Forastero, Criollo, and Trinitario. The first comprises 95% of the world production of cacao, and is the most widely used. Overall, the highest quality of cacao comes from the Criollo variety and is considered a delicacy; however, Criollo is harder to produce, hence very few countries produce it. The Trinitario is a mix between Criollo and Forastero (Ccbolgroup [Online], 2007).

Cacao is cultivated for its seeds, which are used in the manufacture of cacao, chocolate, cacao butter, etc. Cacao seeds are the source of commercial cocoa, chocolate, and cocoa butter. Fermented seeds are roasted, cracked and ground to give a powdery mass from which fat is expressed. Cacao butter is used in the manufacture of confections, toilet preparations, and cosmetic ointments, and in pharmacy or coating pills and preparing suppositories.

Wehmer (as cited in bpi.da.gov. [Online], 2009) reported that the leaves contain an alkaloid, theobromine, caffeine, etc. The wall and pulp of the fruit contain arabinose and galactose; the pulpy flesh; the enzymes: protease: invertase, raffinase, cesease and oxidase. The mesocarp and seed contain theobromine and caffeine. The seeds contain 40 to 56 percent of fixed oil, cellulose 2.8 to 5.4 percent, water 5 to 7 percent, ash 3 to 5 percent, glucose 0.27 to 0.46 percent, saccharose 0.50 to 98 percent, starch 5 percent, etc.; and a glucoside, cacarine ($C_{16}H_{20}O_6N_8$).

Cocoa beans contain 10,000 milligrams (10 grams) of flavanol antioxidants per 100 grams - or an amazing 10% antioxidant concentration level. The antioxidants in cocoa are easily absorbed by the human body, and are more stable and long-lasting than those in any other foods. Flavanols help make blood platelets less likely to stick together and cause blood clots, heart attacks, and strokes - without the negative side effects associated with the use of aspirin (ASA) and other pharmaceutical blood-thinners. Cacao contains oleic acid, a heart-healthy monounsaturated fat which is also found in olive oil and is believed to raise the level of the "good cholesterol" known as HDL cholesterol.

Cacao is reported to be antiseptic, diuretic, ecbolic(promotes labor by stimulating uterine contractions), emmenagogue (promotes or stimulates menstrual flow), and parasiticide, Cacao is a folk remedy for alopecia, burns, cough, dry lips, eyes, fever, listlessness, malaria, nephrosis, parturition, pregnancy, rheumatism, snakebite, and wounds. Cocoa also appears to have anti-aging and anti-inflammatory properties. Cocoa butter is applied to wrinkles in the hope of correcting them (Purdue [Online], 2007). Cocoa has a high content of the "beauty" mineral, sulfur. Sulfur helps build strong nails and hair, promotes healthy and beautiful skin, helps detoxify the liver, and supports healthy functioning of the pancreas. Cocoa is also a good source of the minerals magnesium, calcium, iron, zinc, copper, potassium, and manganese plus some of the B Vitamins (Astrologyzine [Online], 2009).

According to Guerrero (as cited in bpi.da.gov. [Online], 2009), in the Philippines, a decoction brewed from the root of cacao is an emmenagogue and is regarded an embolic. Grieve (as cited in bpi. da.gov. [Online], 2009) on the other hand affirms that the oil of Theobroma or cacao butter is an excellent emollient and is used to soften and protect chapped hands and lips. Theobromine resembles caffeine in its action, but its effect on the central nervous system is less powerful. Its action on muscles, the kidney, and heart is more pronounced. It is used principally for its diuretic effect, which is due to stimulation of the renal epithelium; it is especially useful when there is an accumulation of fluid in the body resulting from cardiac failure, when it is often given with digitalis or relieves dilatation. It is also employed in high blood pressure, as it dilates the blood vessels. It is best administered in powders or cachets. It is also used in eczema and in dry skin where seeds are roasted and pounded which are then applied to affected areas as poultice after a warm compress. Cocoa butter (oil of Theobroma cacao) is an excellent emollient for use to prevent chapped lips and hands. It is also used in the manufacture of confections toilet articles and cosmetics. In pharmacy, it is used for pill coating and suppository preparation (Stuartxchange [Online], 2009). Its bacterial activity however is not firmly established by scientific research other than by folkloric sources.

OBJECTIVES OF THE STUDY

This research aims to assess the antibacterial properties of cacao. Moreover, the study intends to determine the chemical compounds present in cacao through phyto-chemical analysis, as well as to describe the in vitro activity, minimum inhibitory concentration, minimum bacterial concentration, and stability as to pH and temperature of cacao crude extract.

MATERIALS AND METHODS

About a kilo of half-ripe cacao fruits were collected from a single tree in the morning and cleaned. The fruits were kept in a sterile container and comminuted in a blender. They were identified and authenticated by a botanist at the National Museum. The pulp samples were kept in a sterile container and were comminuted in a blender. About 500 g of the comminuted cacao pulp was weighed in a 1000 ml Erlenmeyer flask which was added with 95% ethyl alcohol. The cacao pure extract was obtained by extracting its juice with the use of clean cloth which was the placed in a clean 500 ml Erlenmeyer flask. The ethyl alcohol in the mixture was allowed to evaporate under the laminar flow. The residue was immersed again in 95% ethyl alcohol and again evaporated in laminar flow. 10mg of cacao crude extract was dissolved in 100 ml of Nutrient broth to give a final concentration of 4000 μg/ml. This was used as the standard stock solution. It was placed in a flask and was covered with aluminum foil to avoid the contamination of the crude extract.

Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as test organisms for the microbiological assays. These were obtained from Mrs. Grace Alma Paredero of Batangas Regional Hospital.

Approximately 15 ml of melted MHA agar was poured into dry and sterile Petri dishes. The medium was then set aside to solidify. A sterile cotton swab was moistened into the test organism suspension. The test organism was aseptically swabbed onto the solidified MHA agar plate by streaking the swab over the entire surface of the agar plates three times, rotating the plate approximately 60 degrees after

each application to ensure an even distribution of the inoculum on the surface of the medium. The swabbed plates were allowed to stand for 5 minutes.

The biological activity of cacao pulp crude extract to the test microorganisms was first determined using the paper disc diffusion method In this method, the cacao pulp crude extract suspension was incorporated onto 6 mm paper discs then gently placed on the seeded assay plates. The zone of inhibition is measured after the incubation. Likewise, a twofold dilution was used to determine the tube with the lowest concentration of cacao crude extract at which no growth is observed was reported as the MIC of the cacao extract against selected bacterial isolates. The tube with the lowest concentration of cacao pulp crude extract that gave no visible growth and the succeeding tubes with no visible growth from the MIC determination with S. aureus ATCC 25923, E. coli ATCC 25922 and P. aeruginosa ATCC 27853 will be further cultured to determine the MBC. For the determination of the stability, cacao pulp crude extract, will be tested against the test organisms with a concentration equivalent to its MIC, at varying pH of 2, 4, 6 and 8 and temperature.

RESULTS AND DISCUSSION

From the 250 grams of fresh cacao pulp, 0.96 grams of crude extract were obtained after ethanolic extraction and evaporation. The percentage yield was 0.39%.

Screening for the Presence of antibacterial Activity of Cacao Pulp crude extract

The activity of cacao pulp crude extract was examined against selected bacterial isolates. Using the disk diffusion method, its antibacterial activity was screened using 4000 μg/ml of cacao pulp crude extract. Table 1 shows the result of the screening for the antibacterial activity of cacao pulp crude extract.

Table 1. Screening for the antibacterial activity of cacao pulp crude extract against selected bacterial isolates

Test Organism	Zone of Inhibition	Interpretation
Staphylococcus aureus ATCC 25923	6 mm	Inactive
Escherichia coli ATCC 25922	6 mm	Inactive
Pseudomonas aeruginosa ATCC 27853	6 mm	Inactive

No zone of inhibition against S. aureus ATCC 25923, E. coli ATCC 25922 and P. aeruginosa ATCC 27853 was observed in all the assay plates as shown in Figure 4. This indicates that cacao pulp crude extract is inactive against selected bacterial isolates at a concentration of 4000 μg/ml. The lack of activity of cacao pulp crude extract against the test isolates may be due to the low concentration of the extract in the impregnated paper discs. It is also possible that the cacao pulp has no active antibacterial component or the ethanolic crude extraction may have failed to extract the said active component.

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) refers to the lowest concentration of antibacterial agent that inhibits the visible growth of a microorganism after overnight incubation (Wikipedia [Online], 2007). Since there is no activity observed in the biological screening of the cacao pulp crude extract against selected bacterial isolates, the MIC was not performed.

Minimum Bactericidal Concentration (MBC)

Minimum Bactericidal Concentration (MBC) refers to the lowest concentration of antibacterial agent required to kill an organism (Wikipedia [Online], 2007). Since cacao pulp crude extract has no activity against the test isolates, the MBC determination was not performed.

Stability of Lemongrass Essential Oil

Stability tests are done to assess the stability of an extract when subjected to several variables like pH and temperature (Guevarra,

2005). In this study however, stability tests were not done because the cacao pulp crude extract is inactive against the test isolates.

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