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Plant Extracts: Bactericidal Agents Against *Escherichia Coli*

Rolime Grace Okias[1], Mark Marvin A. Roa[1], Joeylene Plaza[1], Jether P. Sumpo[2]

[1]Secondary Education Department, College of Education, [2]Natural Sciences and Mathematics Department, College of Arts and Sciences Mindanao State University Buug, Datu Panas, Buug, Zamboanga Sibugay, Philippines
Email: rolimegrace17@gmail.com, jether.sumpo@msubuug.edu.ph

ABSTRACT

Escherichia coli is a gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* commonly found in the lower intestine of warm-blooded organisms. A study was conducted to determine the significant difference on the bactericidal effect of various plant extracts on the *Escherichia coli* in terms of the zone of exclusion. The treatments used are the following: distilled water, calamondin extract, garlic extract, gotu kola extract; and combination of all extracts. The application of the treatments was done through Disk-diffusion Method. Disks of filter paper were impregnated with a specific plant extract and placed on the agar plate that have been previously inoculated with *E. coli*. Data were collected by measuring the diameter of the clear zone in terms of millimeters. Based on the results of the study, there is a significant difference on the bactericidal effect of various plant extracts on *E. coli* and that the calamondin extract shows the greatest inhibitory effect on the growth of *Escherichia coli.* This is maybe because calamondin extract contains sinapic acid which shows antioxidant, antimicrobial, anti-inflammatory, anticancer, and anti-anxiety activity (Niciforovic and Abramovic, 2014). Based on the conclusions made, it is recommended that calamondin extract could be used as treatment for *E. coli* related infections.

Keywords: bioassay, bactericidal effect, diffusion method, zone of exclusion

1. INTRODUCTION

Escherichia coli is a gram-negative, facultative anaerobic and non-sporulating bacterium that is a member of the family *Enterobacteriaceae* and is commonly found in the lower intestine of warm-blooded organisms. Most strains are harmless, but some serotypes such as the *E. coli* O157:H7-produces a powerful toxin that damages the lining of the small intestine which can cause bloody diarrhea. Globally, it is the most common cause of urinary tract infections and a leading cause of bacteremia and neonatal meningitis, kidney failure abdominal cramps, bloody diarrhea and vomiting. Signs and symptoms of *E. coli* O157:H7 infection typically begin three or four days after exposure to the said bacteria. Unlike many other disease-causing bacteria, *E. coli* can cause an infection even if you ingest only in small amounts (Felson, 2018).

As reported by the World Health Organization (WHO), at least 600 million people suffer from such illness each year.

The prevalence of *Escherichia coli* in Malaysia ranged from 22.6 to 88.0%. Majority of the work on e coli is concentrated on beef samples. Most Malaysians eat out therefore, posing a high risk of ingesting pathogenic *E. coli* strains. A repost on the prevalence and antibiotic resistance of *E. coli* strains in Malaysia is necessary to create more awareness of the existence of the pathogen in the food chain and its implication on public health (Frederick, 2011).

Meanwhile, a total of 9599 isolates of gram-negative bacteria causing urinary tract infection (UTI) were collected from 60 centres in 13 countries in Asia-Pacific region from 2010-2013. The isolates comprised Enterobacteriaceae species mainly *E. coli*, *Klebsiella pneumonia*, *Proteusmirabilis*, *Kleibsiella oxytoca*, *Enterobacter cloacea* and *Morganella morganii*. In vitro susceptibility profiles were determined using the minimum inhibitory concentration (MIC) interpretive breakpoints recommended by

the Clinical and Laboratory Standards Institute in 2015. It was found that China, Vietnam, India, Thailand and Philippines had the highest rates of Gram-negative bacteria species producing ESBLs and the highest cephalosporin resistance. Antibiotic resistance is a serious problem in the Asia-Pacific region. Therefore, continuous monitoring on the evolutionary trends in the susceptibility profiles of Gram-negative causing UTIs in Asia is crucial (Jean, 2016).

Travel to Asia is a strong predictor for carriage of cephalosporin resistant *E. coli* and *Kleibsiella spp*., according to a prevalence study at a Norweigian hospital on 2014-2016. Of 747 patients, 45 (6.0%) were colonized with ESCr-EK. The ESCr-EK isolates in 41 patients were multidrug resistant; travel to Asia was a strong predictor for colonization of ESCr-EK was being considered when administering empirical antimicrobial treatment. As less than one third of colonized patients had travelled to Asia, and no other factors investigated were found to be strongly associated with carriage, the findings underscore that a healthcare personnel must apply standard infection control precautions for all patients (Espenhain et al, 2018).

In the Philippines alone, over 32,000 cases of food and waterborne disease have been recorded by the Department of Health (DOH) as of September this year. One major culprit for food poisoning is a common type of bacteria called *Escherichia coli* or simply known as *E. coli* (SGS Philippines, 2017).

Ten people died including an 11-month infant in a Diarrhea outbreak in Balabac, Palawan. Authorities blamed the outbreak on contaminated drinking water that tested positive for coliform and poor sanitation due to the presence of *E. coli* from human and animal waste in the town's water table. According to the provincial health officer Mary Ann Navarro, almost all water sources of Balabac proper are not fit for drinking after tested positive to coliform. In September 2017, a diarrhea outbreak also hit Quezon town in Palawan for the same cause. It was reported that four people died and over 600 people were hospitalized at that time (Xinhua, 2018).

The infection caused by *Escherichia coli* is treated mostly by the use of synthetic pharmaceutical products to inhibit the growth of bacteria but can harm the kidneys in different ways like producing crystals that doesn't dissolve in urine thus blocking the flow of urine which can cause kidney swelling and inflammation. An estimated 20% of cases of acute kidney failure are due to medication. There are also circumstances of the unpleasant side effects and increasing resistance of *Escherichia coli* and other bacteria to synthetic pharmaceutical products (Rodriguez, 2017).

The plant extracts that have been used in the study are being known to have antibacterial properties and they were concentrated through steam bathing before applying as treatments. The sources of the treatments are native in our country and can be easily obtained. Perhaps in the last three decades pharmacological industries have produced a massive number of new antibiotics. Its importance cannot be overemphasized in our era of emerging resistant pathogenic organisms. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. For generations, plants have been a valuable source of natural products for maintaining human health. The use of plant extracts and phytochemicals, both with known microbial properties can be of great significance in therapeutic treatments.

Calamondin (*Citrus microcarpa*) is an economically important citrus hybrid predominantly cultivated in the Philippines. The plant is characterized by wing-like appendages on the leaf petioles and white or purplish flowers. The fruit of calamondin resembles a small, round lime usually 25-35mm (0.98-1.38 in) in diameter, but sometimes up to 45 mm (1.8 in). The center pulp and juice is the orange color of a tangerine with a very thin orange peel when ripe. Each fruit contains 8 to 12 seeds. Since Middle Ages its essential oils has been widely used for their bacterial, virucidal, fungicidal, anti-parasitical, insecticidal, medicinal and cosmetic proprieties.

Citrus fruits like calamondin are known to contain bioactive compounds such as phenolic, flavonoids, vitamins, and essential oils which are believed to be responsible for a range of protective health benefits including ant oxidative, anti-inflammatory, antitumor, and antimicrobial activities (Aruoma et al, 2012).

The fruits are sour and often used in preservation and cooking. Calamondin is a fruit that is more than meets the eye. The pectin content found in the pulp of the fruit is known to work wonders in lowering blood cholesterol. Calamondin has several alternative medicinal uses. The most popular medicinal use of calamondin is when taken orally as a cough remedy. Other races like Malays combine pepper to help expel the phlegm. It is also used in skin and hair care products. The flavonoids from *Citrus* exhibit in vitro and vivo anti-inflammatory, anti-cancer, antioxidant and cardiovascular protective activities (Dulay et al, 2016)

Garlic (Allium sativum) has long been a common use in culinary and traditional medicine. With a history of several thousands of years of consumption and use. Its close relatives include the onion, shallot, leek, chive, and Chinese onion. Garlic is native to Central Asia and north-eastern Iran, and has long been a common seasoning worldwide, with a history of several thousand years of human consumption and use It was known to ancient Egyptians, and has been used both as a food flavoring and as a medicine garlic is used for many conditions related to the heart and blood system. These conditions include high blood pressure, low blood pressure, high cholesterol, inherited high cholesterol, coronary heart disease, heart attack, reduced blood flow due to narrowed arteries, and "hardening of the arteries" (atherosclerosis).

Garlic produces a chemical called allicin. This is what seems to make garlic work for certain conditions. Allicin is a compound produced when garlic is crushed or chopped. Available in dietary supplement form, its ben found to reduce inflammation and offer antioxidant benefits (Wong, 2019)

Garlic's potential to combat heart disease has received a lot of attention, but it should receive even more acclaim for its antimicrobial properties. Fresh, raw garlic has proven itself since ancient times as an effective killer of bacteria and viruses. Once again, we can thank allicin. Laboratory studies confirm that raw garlic has antibacterial and antiviral properties. Not only does it knock out many common cold and flu viruses but its effective-

ness also spans a broad range of both gram-positive and gram-negative bacteria (two major classifications of bacteria), fungus, intestinal parasites, and yeast. Cooking garlic, however, destroys the allicin, so it needs to be used as raw to prevent or fight infections.

One of the active principles of freshly crushed garlic homogenates, has a variety of antimicrobial activities. Allicin its pure form exhibits antibacterial activity against a wide range of gram positive and gram- negative bacteria, fungus, intestinal parasites, and yeast. The main antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin ructase, and RNA polymerase (Ankri, 1999).

Gotu kola also known as *Centella asiatica* is an herbaceous, frost-tender perennial plant in the flowering plant family of *Apiaceae*. It is native to the wetlands in Asia. It is used in culinary and medicinal purposes. The centella plant stems are slender, creeping stolon, green to reddish green in color, connecting plants to each other. It also has a long-stalked green, rounded apices which have smooth texture with palmately netted veins. Its flowers are white or pinkish to red in color. They are small with rounded umbels near the surface of the soil.in traditional medicine, *C. asiatica* has been used to treat various disorders and minor wounds. Apart from wound healing, the herb is recommended for the treatment of various skin conditions such as leprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhea, fever, amenorrhea, anxiety and cognition. It has been used for decades in South-east Asia and India for skin conditions, wound healing and memory improvement.

The primary constituents of *C. asiatica* are saponins or triterpenoids. These triterpene saponins and its sapogenins are mainly responsible for wound healing (Gohil, 2016).

Analyses of the essential oil of gotu kola medicinal plant revealed 11 monoterpenoid hydrocarbons (20.20%), nine oxygenated monoterpenoids (5.46%), 14 sesquiterpenoid hydrocarbons (68.80%), five oxygenated sesquiterpenoid (3.90%), and one sulphide sesquiterpenoid (0.76%). humulene, caryophyllene, bicycle germacrene, germacrene B and myrcene were the predominant constitutes. The essesntial oil extract exhibited broad spectrum of antibacterial activities against Gram positive and Gram-negative bacteria such as *E. coli* and other organisms. (O. Oyedeji, 2005) .It also possess anti-microbial, anti-inflammatory activity, it can be effectively used as a prophylactic and therapeutic agent against bacteria which can be easily available and economical (Vijayashree, 2015).

OBJECTIVES

This research aimed at evaluating the bactericidal property of calamondin, garlic and gotu kola extracts on the growth of gram-negative bacterium, *Escherichia coli* and to distinguish which of the following extracts show the greatest inhibitory effect in the said bacteria.

2. METHODOLOGY

Preparation of the Pure Culture of Escherichia coli. The *E. coli* was inoculated to a beaker containing natural saline solution at 40ml. Using a cotton applicator, the sample from the beaker was streaked in the prepared media. Then, the culture was incubated to allow the growth of bacteria. After which, inspections were done to check if there are colonies of *Escherichia coli.*

The colony of *Escherichia coli* present in the medium was transferred to new medium through the use of inoculating loop into another beaker containing natural saline solution. Using a cotton applicator the sample from the beaker was streaked in the prepared media. Then, it was incubated for growth. This process was repeated until a pure culture is produced.

Preparation of the Plant Extracts. Six (6) calamondin fruits were washed thoroughly with water and were sliced into halves. After that, the fruits were squeezed in the pitcher. Then the juice was strained in order to remove the seed. Then, the calamondin extract was stored in a sterilized beaker and is set aside for the treatments.

At least three pieces of garlic were peeled and were washed thoroughly with water and were then dried. After drying, the garlic cloves were placed on a juicer for extraction of the juice. The garlic extract was stored in a sterilized beaker and was set aside for the treatments.

A good amount of gotu kola leaves were washed thoroughly with pre-heated water to remove dirt. Then, the leaves were allowed to dry. After drying, the leaves of the gotu kola were placed in the juicer to extract the juice. The juice was strained to remove the tiny pulp from the leaves. The gotu kola extract was then stored in a sterilized beaker and was kept being used in treatments.

All of the extracts undergo steam bathing to remove excess water from the extracts and to have a concentrated amount of extracts from 10ml to 5ml.

Preparation of the Culture Media. Eleven and four (11.4) grams of Mueller Hinton agar was measured using the triple beam balance. Then, it was dissolved in 300ml distilled water in a beaker. The agar was heated in a stove and was stirred constantly until near boiling and completely dissolved. Then, the beaker with the cooked Mueller Hinton agar was covered with aluminum foil. The covered cooked nutrient agar and laboratory apparatuses such as the fifteen (15) petri dishes, natural saline solution, beakers, cotton applicators, filter paper and loop dilution rod were placed in the autoclave. The autoclave was pressurized for ten (10) minutes and was depressurized. Then, the autoclave was set again to fifteen (15) *psi* at 120°c and maintained in this pressure and temperature for fifteen (15) minutes. Then, the autoclave was depressurized and was allowed to cool. The nutrient agar was carefully placed in every sterilized petri dish which was done over the heat of a flame of an alcohol lamp. Each petri dish contains 20ml of the cook agar media. The petri dish was covered and the Mueller Hinton agar was allowed to solidify.

Culturing of the Research Specimen. A good amount of *Escherichia coli* was taken from the pure culture using the inoculating loop and was dispersed in 40 ml natural saline solution. With the use of a cotton applicator, the *Escherichia coli* was spread over the solidified Mueller Hinton agar in the prepared media. This was also done over an alcohol lamp in order to have

a pure culture. Finally, the prepared culture was set aside and allowed to grow for twenty-four (24) hours.

Experimental Design. There were five (5) treatments and each treatment have three (3) trials which serve as replicates, having a total of fifteen (15) culture media.

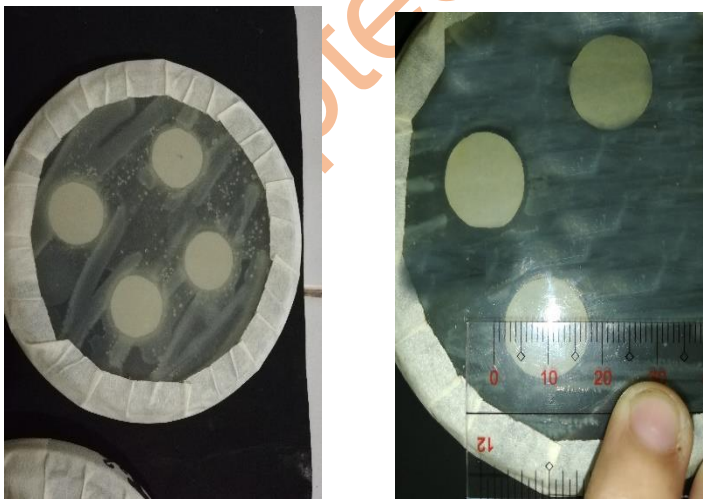
Application of treatments. When the incubation period of twenty-four (24) hours of the pure culture of the *Escherichia coli* ends, the fifteen (15) different petri dishes were grouped into five (5), with three (3) petri dishes in every group. The first group was treated with distilled water, the second group was treated with the calamondin extract, the third group was treated with the garlic extract, the fourth group was treated with the gotu kola extract, and the fifth group was treated with the combination of the three (3) plant extracts.

The application of the treatments was done through Kirby-Bauer disk-diffusion method. Disks of filter paper were soaked with the extracts which was previously prepared in the sterilized beakers. Each beaker with a treatment contains at least twelve pieces of disk. Four pieces of disks were placed in every culture media having a total of twelve disks applied in every group. The treatments were applied only once. Finally, the culture media was incubated to allow the effects of the treatments to occur on *E. coli*.

Data Collection. The first set of data were collected on the 8th hour, second data set were collected on the 16th hour, and the third data set were collected on the 24th hour.

The diameter of the zone of inhibition (in mm) was measured using a plastic ruler. The diameter of the filter paper disk (14 mm) was included to the measured diameter of the zone of exclusion. The average measures of the four (4) zones of exclusion found around the four disks were computed and treated statistically.

Figure 1 shows the photos taken after the treatment applications (a) and eight hours after treatment application (b).



a b

Figure 1. (a) Treated Microbial Plate, (b) Plate showing Zone of Inhibition

3. RESULTS AND DISCUSSION

The figures below show the results of the average zones of exclusions at the 8th, 16th, and 24th hours after treatment applications.

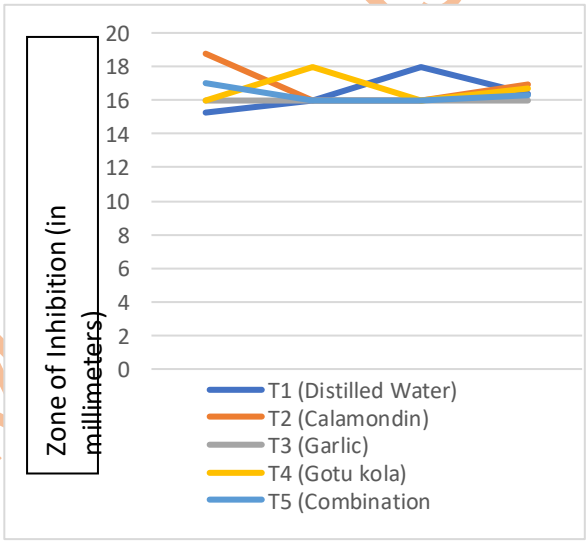


Figure 2. Average zone of exclusion at the 8th HATA.

Figure 2 shows the average zone of exclusion at the 8th hour after treatment application (HATA). Treatment two (T_2) which is the calamondin extract shows the greatest inhibitory effect with a mean of 16.92 mm followed by Treatment four (T_4) at 16.67mm of which is the gotu kola. The size of the disks are 14mm.

ANOVA revealed that there is no significant difference on the bactericidal effect of plant extracts on *E. coli* at the 8th HATA.

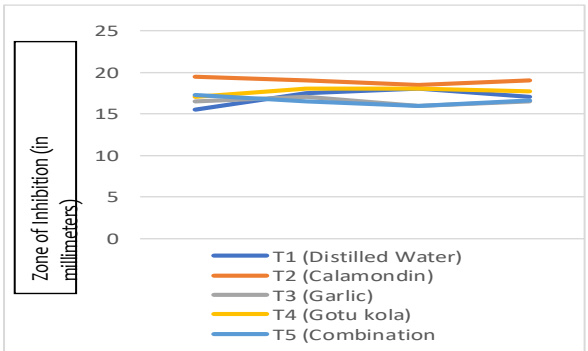


Figure 3. Average zone of exclusion at the 16th HATA.

Figure 3 shows the average zone of exclusion at the 16th hour after treatment application (HATA). Treatment two (T_2) which is calamodin extract showed the greatest inhibitory effect with the mean of 19.00mm followed by Treatment four (T_4) at 17.67mm which is the gotu kola.

ANOVA revealed that there is a highly significant difference on the bactericidal effect of plant extracts on *E. coli*. The Duncan's Multiple Range Test (DMRT) further revealed that treatment two (T_2), the calamondin extract showed the best inhibitory effect on *E. coli.*

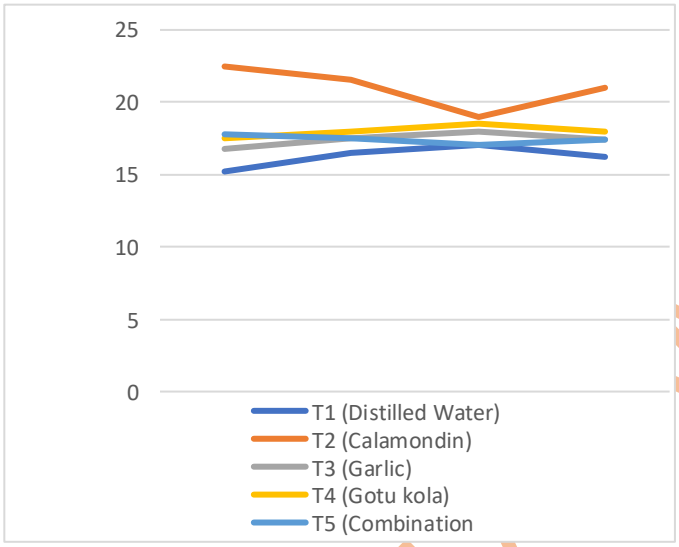


Figure 4. Average zone of exclusion at the 24th HATA.

Figure 4 shows the average zone of exclusion in the 24th hour after treatment application (HATA). Treatment two (T_2) which is calamondin extract shows the greatest inhibitory effect with a mean of 21.00mm followed by Treatment four (T_4) at 18.00mm which is gotu kola extract.

ANOVA revealed that there is a highly significant difference on the bactericidal effect of the plant extracts. The Duncan's Multiple Range Test (DMRT) further revealed that treatment two (T_2) the calamondin extract showed the best inhibitory effect on *E. coli*.

This is probably due to the sinapic acid of calamondin extract which caused the greatest inhibitory effect among other treatments.

The results of the study also implicate that the inhibitory strength of sinapic acid is observed almost immediately after treatment application. The data in Figure 4 shows that the inhibitory strength of the plant extracts is at its highest on the 24th HATA.

4. CONCLUSIONS

Based on the results of the study, it can be concluded that there is a significant difference on the bactericidal effect of various plant extracts on *Escherichia coli*. Moreover, it can be concluded that Treatment two (T_2) which is the calamondin extract shows the greatest bactericidal effect on the growth of *Escherichia coli.*

TRANSLATIONAL RESEARCH

Rolime Grace and her colleagues at Mindanao State University-Buug Campus found out that among the five treatments which is the water, calamondin, garlic, gotu kola and combination of all extracts; calamondin extract shows the greatest inhibitory effect in the course of 24 hours, to the bacterium *Escherichia coli*, a gram-negative, facultative anaerobic, rod-shaped, coliform bacterium commonly found in the lower intestine of warm-blooded organisms which is the most common cause of urinary tract infections and a leading cause of bacteremia and neonatal meningitis, kidney failure abdominal cramps, bloody diarrhea and vomiting. Calamondin extract could be used in the food industry to prevent contamination from *E. coli* and treating other gram-negative bacteria.

5. RECOMMENDATIONS

Based on the results of the study, the calamondin extract is recommended as treatment against infections caused by *E. coli*. In salads and ceviche, it is better to add calamondin not just to inhibit the growth of *E. coli* but it can also boost immunity and metabolism, control cholesterol levels, improve respiratory health, lower acidity, promote collagen production and helps detoxify the body. Since the Philippines is the number one producer of calamondin, we might synthesize medicine from it. In relation to calamondin, it is recommended to use other parts like the leaves and stem rather than the juice itself. Moreover, it is recommended that a follow up study and experimentation be conducted on the Bactericidal Effects of various Plant extracts on *E. coli.*

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