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Study of antimicrobial activity of aqueous and alcoholic *Eugenia caryophyllata* extracts on *E.coli* bacteria isolated from Diarrhea in children under 5 years in Tikrit city

Ala'a Abdul Jabbar Jameel¹ and Ahmed Hashim Alanee²

¹Dept. of Microbiology, College of Science, Tikrit University.

²Dept. of Pediatrics, Tikrit Medical College.

*Corresponding author: E-mail: ah70.tucam@tu.edu.iq

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KEY WORDS:

Eugenia caryophyllata extracts, *E.coli*, diarrhea, antibiotics

ABSTRACT

Background: Diarrheal disease is common among children worldwide, especially in developing countries.

Aims of study: To Test the inhibitory effectiveness of aqueous and alcoholic *Eugenia caryophyllata* extracts on *E.coli* which were isolated from young children under five years with diarrhea.

Materials & Methods: One hundred samples were collected from children with diarrhea under five years. 75 isolates of *E.coli* were isolated and diagnosed based on their phenotypic properties and biochemical tests.

Results and Discussio: The results of the susceptibility test to 13 types of antibiotics using the disc diffusion method showed a variation in the level of resistance to the different antibiotics, and it was found that the most effective antibiotics were Imipenem and Nitrofurantoin. The study used aqueous and alcoholic extracts of *Eugenia caryophyllata* seeds and was tested for their ability to inhibit the growth of *E. coli*

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INTRODUCTION

Infant diarrhea is a common cause of death for children less than five years .⁽¹⁾ The causes of diarrhea are different types, which may be viral, bacteriological, or parasitic. They are found about 20% of the children infected by bacterial of *Campylobacter jejuni* , *Yersinia*, *Salmonella*, *Shigella*, pathogenic *E. coli*, and *Clostridium difficile*⁽²⁾. Most strains of *E.coli* live in the intestine and rarely cause disease in healthy individuals. However, several strains cause diarrhea or extra-intestinal illness in both healthy and immunocompromised individuals. Diarrheal diseases are a severe public health problem and a major cause of Pathogenicity and death in infants and young children, especially in developing countries. The strains of *E.coli* bacteria that cause diarrhea have evolved by transferring a set of genes that have properties that enable them to persist persistently within the host. These strains are now known as DEC (Diarrheagenic *E.coli*)⁽³⁾. These strains have been divided into six main categories based on Characters epidemiological and clinical features and specific virulence factors in association with some serotypes, including: - enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC), enteroinvasive *E.coli* (EIEC), enterohemorrhagic *E.coli* (EHEC) or Shiga-toxin producing *E.coli* (STEC), enteroaggregative *E.coli* (EAEC), diffusely adherent *E.coli* (DAEC)⁽⁴⁾. enteropathogenic *E.coli* (EPEC) is an important cause of diarrhea in infants in developing countries and has been historically identified based on serotypes such as O55:H6 and O127:H6. This pathogen works to disrupt the function of intestinal epithelial cells by attaching and effacing (A/E), as These pathogens are characterized by their local destruction of microvilli and the formation of a lectin-rich structure that helps them adhere to host cells. Another virulence factor is that it possesses large portions of plasma in common with Enterotoxigenic *E.coli* O157:H7 strains. EPEC causes disease by growing in the small intestine, causing watery, mucous diarrhea without blood, vomiting, fever, and dehydration. Symptoms usually last for several days despite long chronic cases⁽⁵⁾.

Eugenia caryophyllata belongs to the family, Caryophyllaceae.⁽⁶⁾ as it was found that Eugenia extract possesses several effective compounds that have an antioxidant and anti-tumor effect. Eugenol, which is the active compound of the extract, has a high bacterial inhibitory activity and

anti-inflammatory activity. It was also shown that Eugenia works to reduce the levels of liver enzymes in the serum. This effectiveness is due to the presence of antioxidants in Eugenia. It also contains phenolic compounds that act as free radical scavengers⁽⁷⁾. The chemical components of the Eugenia plant include eugenol, caryophyllene, and tannins, and it contains 14%-20%. Volatile oils and small amounts of tannins, sitosterol, stigmasterol⁽⁸⁾.

Materials and Methods

Preparation of culture media: Culture media were prepared according to the manufacturer's instructions. The Culture media were sterilized at a temperature of 121 ° C and pressure of 15 lb / kg for 15 min. After That, incubate at 37 ° C for 24 hours before use to make sure it is not contaminated Until use⁽⁹⁾.

Collection of Samples: One hundred samples were collected from children with diarrhea under five years in the emergency of Salah Alden General Hospital for the period from the beginning of August 2022 to the end of December 2022. These samples were collected in sterile plastic bottles. A child who had taken antibiotics before entering Hospital emergency was excluded from the study.

Culturing of Sample: Samples were cultured on the MacConkey agar and EMB agar, and incubated at 37 ° C for 24 hours. The developing colonies were then purified to obtain individual colonies for diagnosis⁽¹⁰⁾.

Diagnosis of bacteria Analyting Profile Index (API 20 E) : The tape contains 20 wells containing dehydrate substrates to detect enzymatic activity usually related to the fermentation of carbohydrate or catabolism of proteins or amino acids by the inoculated organisms. Then a homogeneous suspension consisting of a saline solution and a bacteria to be diagnosed was transferred to 0.12 ml of bacterial suspension for each test tube and 0.28 ml of suspension for GEL, VP, CIT, and 0.1 ml oil for the ODC, LDC, ADH, URE, H₂S Provide anaerobic conditions. The reagents were added to the incubator at 37 ° C for 24 hours. The reagents were added according to the requirements of each test and the results were read, turn the results were turned into numbers and then compared with the codes in the index supplied by the company (Bio-Merieux / France) to give the name of the species and type of bacteria.



Figure (1) : API 20 E supplied by the manufacturer (Bio-Merieux / France)

Antibiotic Susceptibility Test of Bacterial Isolates:

The antibiotic Susceptibility test was conducted on the Muller Hinton agar using the antibiotics prescribed in Table 1. A single colony was transferred into tubes containing 5 mL of the Nutrient broth and incubated at 37°C for 24 hours, and then turbid growth with turbid constant

solution standard turbid (McFarland solution). The bacteria were cultured to the Muller Hinton agar using cotton swab and the discs were placed on the plate using sterile forceps, and incubated at 37°C for 24 hours. Then the inhibition zone was measured⁽¹¹⁾.

Table (1): Types of antibiotics used in the study and their concentration (micrograms/tablet)

| | Antibiotics | Symbol | Concentration µg/disc |
|----|--------------------------------|--------|--------------------------|
| 1 | Nitrofurantoin | NI | 300 |
| 2 | Cefixime | CFM | 5 |
| 3 | Trimethoprim- Sulfamethoxazole | TS | 1.25 /23.75 |
| 4 | Penicillin-G | P2 | 10 |
| 5 | Amoxycillin | AMX | 30 |
| 6 | Nalidixic Acid | NA | 30 |
| 7 | Cepodoxime | CPD | 10 |
| 8 | Cefotaxime/clavulanic acid | CEC | 30/10 |
| 9 | Cloxacillin | CX | 5 |
| 10 | Gentamicin | GM | 10 |
| 11 | Imipenem | IMI | 10 |
| 12 | Ampicillin | SAM | 10 |
| 13 | Cefuroxime | CXM | 30 |

Preparation of aqueous extracts

These extracts were prepared for the plants used under study by mixing (40) gm of the plant model in (160) cm³ of distilled water, i.e. a ratio of 4: 1 w / v. The plant model was placed in the blender inside an ice bath, then the model or mixture was stirred by the magnetic stirrer for at least one hour to analyze and tear the plant cell wall. Then the mixture was placed in the refrigerator for 24 hours to soak well and then filtered. The mixture was made through several layers of gauze and then filtered again by a Buchner funnel using Whatman No1 filter paper to remove the remnants of the fibers and unpowdered portions, with this step the raw aqueous extract was prepared, and then the

extract was dried by cooling under rare pressure under lypholizer apparatus, and then put the samples after drying inside glass bottles with tight lids in zero humidity conditions, after that the aqueous plant extracts were preserved by freezing them for use in conducting the study⁽¹²⁾.

Preparation of alcoholic extracts

In the preparation of alcohol extracts, crushing was adopted by (50) gm of the plant used in the research In(200) ml of ethyl alcohol at a concentration of 95% inside an ice-water bath, the plant used in the analysis, after which the mixture was shaken well by the magnetic stirrer device. Keep in the refrigerator for 24 hours, filter the mixture using several layers of gauze, and to

get rid of the alcohol, the mixture is placed in the Rotary Evaporator, which works based on evaporation under low pressure at a temperature not exceeding 40 ° C, then after evaporation The solvent (alcohol) from the mixture is a thick layer of the extract which dried by cooling under rare pressure under lyophilizer apparatus, and then put the samples after drying inside glass bottles with tight lids in zero humidity conditions, after that the alcoholic plant extracts were preserved by freezing them for use in conducting the study ⁽¹²⁾.

Sterilization of aqueous extracts

1 gm of plant extract powder was added to 5 ml of distilled water to produce a standard concentration of 200 mg/ml. It was then sterilized by passing it through a membrane filter, and then the standard concentration was used to prepare the remaining concentrations of 200, 100, 50, 25, and 12.5 mg/ml ⁽¹²⁾.

Sterilization of alcoholic extracts

1 g of plant extract powder was added to 5 ml of ethanol, then sterilized by pasteurization, and the same concentrations mentioned above were prepared ⁽¹²⁾.

Determination of *E.coli* susceptibility to various concentrations of solution clove plant extracts (In vitro):

The Well diffusion method was used by making three iterations according to the method⁽¹²⁾. Then 50 micro-liters of the prepared extract were added to each hole with successive concentrations (200,100,50,25,12.5) mg/ml, and in the standard well, distilled water was placed in the case of aqueous extract, and DMSO solution in the case of alcoholic extract, then And placed in the incubator for 24 hours at a temperature of 37 ° C, the potency of the concentrations of the plant extracts were determined by measuring the diameter of the inhibition zone in mm using a standard ruler.

RESULTS AND DISCUSSION

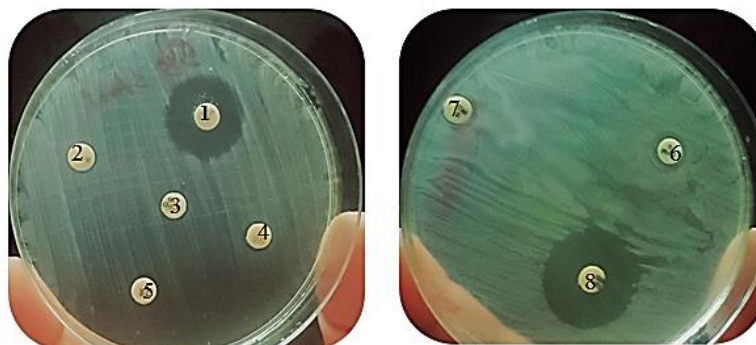
One hundred and fifty stool samples were collected from children with diarrhea under five years of age, and the results showed that (100) samples showed bacterial growth, as 75 (75%) of the isolates were isolated and diagnosed as belonging to the bacteria *E.coli*. The *E.coli* showed pink, dry colonies on MacConkey culture because it ferments the sugar lactose ⁽¹³⁾, while on the EMB medium, the *E.coli* appeared in a bright metallic green color, and this is consistent with the observations of⁽¹⁴⁾.API 20 E system to confirm these results (Figure 2).



Figure (2) API 20 E test results OF *E.coli*

The Muller-Hinton medium was used to determine the antibiotic susceptibility against these isolated bacteria and was interpreted according to ⁽¹¹⁾into susceptible, moderately

sensitive, and resistant. The *E.coli* bacteria isolates showed different resistance patterns to antibiotics, as shown in the figure (3)



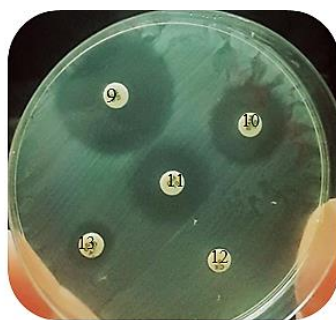


Figure (3) Diameters of inhibition of isolated antibacterial: *E.coli*, 1: Cefotaxime, 2: Amoxycillin, 3: Cefpodoxime, 4: Penicillin-G, 5: Nalidixic Acid, 6: Nitrofurantoin, 7: Trimethoprim, 8 Cefixime, 9: Imipenem, Ampicillin: 10, 11: Gentamicin, 12: Cloxacillin, 13: Cefuroxime

Table (2) Resistance of *E. coli* from children with diarrhea to the antibiotics used in the study. Among 75 isolates of *E.coli*, the percentage of resistance was (100%) to Amoxycillin, Penicillin-G, and Cloxacillin, this study agrees with conducted ⁽¹⁵⁾ in Baghdad ⁽¹⁶⁾ and in Babylon, who found that *E. coli* is 100% resistant to Amoxycillin and Penicillin-G. This study does not agree with ⁽¹⁸⁾ in Baghdad who found resistance to *E.coli* for amoxicillin at a rate of (50%) and (93.3%) for Cefotaxime/clavulanic acid. This agrees with the study of ⁽¹⁹⁾ and does not agree with the study of ⁽¹⁵⁾ which found that the percentage of resistance was (67.5%). *E.coli* isolates were(86.7%) resistant to Cefuroxime, Cefpodoxime, and Trimethoprim-Sulfamethoxazole, and this agrees with ⁽¹⁹⁾, who found that the rate of resistance to Trimethoprim was(89.5%), as well as ⁽²⁰⁾, in Kufa, who found the rate(83. %). *E.coli* was(80%) resistant to Cefixime. The percentage of resistance was(66.7%) for Ampicillin, and this result does

not agree with ⁽¹⁵⁾, which found the percentage (42.5%) and with ⁽¹⁷⁾, who found the percentage (86.5%). The percentage of resistance to Nalidixic Acid was (46.7%). This study agrees with ⁽¹⁵⁾, which found the percentage to be (45%) and does not agree with ⁽²⁰⁾ in Erbil, who recorded a resistance percentage of (14.3%). The percentage of resistance to Gentamicin was (33.3%), which agrees with the study of ⁽¹⁵⁾, which found a resistance rate of (30%), and with ⁽¹⁷⁾, which was (45.4%), and does not agree with both ⁽²¹⁾⁽²²⁾ in Baghdad and Babylon, who found a percentage of (70%) and (50%), respectively. As for the percentage of resistance to Imipenem, it was (13.3%), and this agrees with ⁽¹⁷⁾ who found the percentage of resistance to be (5.5%), and the percentage of resistance to the anti-nitrofurantoin was (6.7%). This result is consistent with ⁽¹⁵⁾ which found the resistance percentage to be (12.5%) and does not agree with ⁽²³⁾ in Libya, which found the percentage of resistance (40%).

Table(2) Resistance of *E.coli* to the antibiotics used in the study

| anti biotic | Number of isolates | Nitrofurantoin | | Cefixime | | Trimethoprim-Sulfamethoxazole | | Gentamicin | | Imipenem | | Ampicillin | | Penicillin-G | |
|---------------|--------------------|----------------|-----|----------------|------|-------------------------------|------|-------------|------|----------------------------|------|------------|------|--------------|-----|
| | | R | % | R | % | R | % | R | % | R | % | R | % | R | % |
| <i>E.Coli</i> | 75 | 15 | 20 | 60 | 80 | 65 | 86.6 | 25 | 33.3 | 10 | 13.3 | 50 | 66.6 | 75 | 100 |
| | | Amoxycillin | | Nalidixic Acid | | Cefpodoxime | | Cloxacillin | | Cefotaxime/clavulanic acid | | Cefuroxime | | | |
| | | R | % | R | % | R | % | R | % | R | % | R | % | | |
| | | 75 | 100 | 35 | 46.6 | 65 | 86.6 | 75 | 100 | 70 | 93.3 | 65 | 86.6 | | |

The results in Table (3) showed the antibiotics most effective in inhibiting *E.coli* were Imipenem and Nitrofurantoin, with diameters of inhibition of 29.3 mm and 20.42 mm. When comparing the rates of diameters of inhibition for antibiotics with the rates of diameters of inhibition for plant extracts (Aqueous *Eugenia caryophyllata*, alcoholic *Eugenia caryophyllata*) It

was found that there were highly significant differences at the probability level (P<0.05) on *E.coli* isolated from diarrhea samples, and it became clear from Table (3) that the extract was most effective in inhibiting the growth of *E.coli* bacteria (Aqueous *Eugenia caryophyllata*) had an average of 17.42 mm. The most effective concentration in inhibiting *E.coli* bacteria was 200

mg/ml, followed by a concentration of 100 mg/ml. The least effective concentration in inhibiting *E.coli* bacteria was 12.5 mg/ml, while at 50 mg/ml and 25 mg/ml, there is no significant difference between them. Aqueous *Eugenia caryophyllata* extract at a concentration of 200 mg/ml was the most effective in inhibiting bacteria, followed by concentrations of 100 mg/ml and 50 mg/ml, as there was no significant difference between the previous two concentrations. Figure (4) The inhibitory effect of plant extracts (*Eugenia caryophyllata* aqueous, *Eugenia caryophyllata* alcoholic).

This study is consistent with what was reported by (24)(28)(29) regarding the high effectiveness of inhibiting *Eugenia caryophyllata* extract against *E.coli*. The ability of the *Eugenia caryophyllata* plant to inhibit various types of bacteria (gram negative, gram positive) is

because it contains Eugenol, a type of phenolic compound that has antimicrobial activity and works to inhibit the mechanism of action of the cellular membrane of microorganisms and thus inhibit the growth of the microorganism (25). Tannins are also among the phenolic compounds that can disrupt the adhesion of bacteria, enzymes, and the transport of cell envelope proteins, while alkaloids can inhibit the growth of bacteria by affecting DNA (26). This study has shown that the inhibitory effectiveness of aqueous extracts is due to the chemical components and minerals contained in them, which interfere with metabolic processes and with the growth of bacteria and thus destroy these bacteria (27).

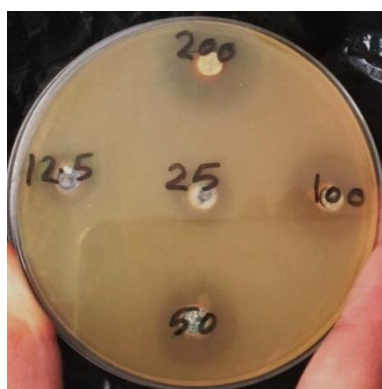


Figure (4-a) (*Eugenia caryophyllata* alcoholic)



Figure (4-b) (*Eugenia caryophyllata* aquatic)

Figure (4) The inhibitory effect of plant extracts (*Eugenia caryophyllata* aqueous, *Eugenia caryophyllata* alcoholic)

Table (3) Inhibitory effect on *E.coli* isolated from diarrhea samples. Similar letters mean that there are no significant differences between them with a probability level of $p \leq 0.05$ according to Duncan's test

| <i>E.coli</i> | mg/ml200 | 100mg/ml | 50mg/ml | 25mg/ml | mg/ml12.5 | middle Abstract |
|--|------------|------------|----------|----------|-----------|-----------------|
| <i>Eugenia caryophyllata</i> aquatic | 21.25b | 18.75cd | 17.5cd | 15.3de | 14.3ef | 17.42 ml A |
| <i>Eugenia caryophyllata</i> alcoholic | 16.8de | 15de | 12.9fg | 11.7fg | 8.25hi | 12.93 ml B |
| Nitrofurantoin | 20.42 bc | | | | | |
| | B | | | | | |
| Imipenem | 29.3 a | | | | | |
| | A | | | | | |
| Average concentration | 16.01 ml C | 14.19 ml D | 12.96 ml | 12.18 ml | 9.81 ml F | |

E E

CONCLUSION

The results of susceptibility tests showed that the most effective antibiotics on *E.coli* for diarrhea cases in children are Imipenem and Nitrofurantoin. The results showed that the plant extract that has the most effective on *E.coli* is the aqueous *Eugenia caryophyllata* extract, followed by the alcoholic *Eugenia caryophyllata* extract.

RECOMMENDATION

- 1- Extracting the active substance of the extracts used (cloves) and conducting a study using laboratory animals to evaluate the role of the active substance within the body of a living organism.
- 2- Study the effect of plant extracts on other species of pathogenic bacteria that infect the digestive system

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