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Synergistic Effect of *Ficus Religiosa* Extract and Ciprofloxacin on Growth and Biofilm Formation of *Escherichia coli* Isolated from Clinical Samples

Adel N. Tomah, Zainab F. Mahmood

Department of Biotechnology, College of Sciences, University of Baghdad

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Abstract: This study aimed to reveal the effect of total flavonoids and crude extract of *ficus religiosa* on biofilm inhibition and to evaluate the synergistic activity of crude and flavonoids with Ciprofloxacin (CIP) as a biofilm antagonist of clinically isolated *E. coli*. Crude extraction was accomplished using 80% methanol and flavonoid extraction accomplished using 100% methanol. 119 clinical urine and stool samples were collected from patients at Medical City Hospital. The Vitik 2 assay identified 76 of them as *E. coli* and only 46 isolates were able to produce biofilm when grown in congo red agar and microtiter plates. Molecular detection was carried out for the availability of genes responsible for biofilm formation in bacterial isolates The bacterial isolates were selected according to their ability to form biofilm and their resistance to antibiotics. The results showed that 21 (70%) isolates possess the *fimH* gene and 17 (56.6%) isolates possess the *pap* for *E. coli*. The results showed that the crude extract was more effective than flavonoids in inhibiting biofilm production at a concentration of 512 µg /ml. The results also showed that the mixture of Ciprofloxacin (8 µg /ml) and the crude extract (128 µg /ml) against biofilm formation of *E. coli* where the percentage of biofilm inhibition was (88%). The flavonoids with Ciprofloxacin against E. coli were (128 µg/ml) and (8 µg/ml), respectively, where the inhibition ratio was (81.1%). At low levels, this showed synergistic efficacy against bacterial biofilm formation. These results show that this drug can be used to fight *E. coli* biofilms well as as an antibacterial drug in the future.

Keywords: Biofilm, HPLC, flavonoid, Crude extract, *fimH and pap gene, Ciprofloxacin.*

Corresponding author: (Email: adilnajah59513@gmail.com).

Introduction

Escherichia coli is the first class of bacterial in urinary infections, moves from the urinary system to the bladder, causing irritation of the bladder, kidneys, ureters, and urethra; kidney inflammation which are more harmful than bladder inflammation. These infections are the second most common bacterial illnesses in the medical community (1,2). The number of people infected with urinary tract infections each year is estimated to be over 150 million (3,4). Biofilms are linked to up to 60% of human diseases and are notoriously hard to get rid of with antibiotics. In vitro susceptibility studies revealed a significant increase in biofilm cell resistance to death (5). Biofilms give bacteria a way to defend themselves by letting them get the most out of the nutrients they have and blocking antimicrobial agents, antibodies, and white blood cells (6). They have a lot of enzymes that make antibiotics not work, like beta-lactamases. This has made them a hot spot for antimicrobial resistance. This study was conducted by (7). Type 1 fimbriae (fim-H) and other genes, such as hemolysin (hly), cytotoxic-necrotizing-factor, and aerobactin, that

make UPEC strains dangerous. Everything contributes to (8). A lot of these things help the organism be able to colonize, evade or subvert the host's defense systems, damage or penetrate host cells, and cause an inflammatory response that leads to clinical disease (9). *Biofilm* production is another virulence mechanism that aids pathogen survival, and bacteria within a biofilm are more resistant to host defense and medications, making infection eradication more difficult. Plant extracts have been found to prevent the formation of bacterial biofilms, motility, adhesion, and cell communication (10) . Plant extracts and their primary components can also reduce the expression of important virulence genes, which suppresses bacterial toxin production (11). Finally, multiple studies have demonstrated that combining plant extracts with antibiotics increases their action while lowering drug doses and side effects. Positive interactions are being looked into as a possible technique for combating bacterial resistance. The synergistic properties of plant extracts and antibiotics will be discussed in the following sections (11).

Materials and methods

Collection of bacterial isolates

Between 17 October and 1 January 2022, One hundred ten clinical pathogenic isolates of stool and urine swab samples were obtained from several hospitals in Baghdad, including Al-Yarmouk Educational Hospital, Al-Kadhmiya Educational Hospital, and Madienat Al-Teb Educational Laboratories. All specimens were streaked on MacConkey agar and Eosin methyl blue (EMB) agar. After that, all plates were incubated aerobically for 24 hrs at37°C.

Procedure for the preparation of crude plant extracts (*ficus religiosa* extract)

Preparation of the crude plant extract (*ficus religiosa* extract) The leaves of *Ficus religiosa* were collected from the garden of Baghdad University Iraq, during the period from October to November 2021, according to (12). a methanolic extract of *Ficus religiosa* was prepared.At 65°C for 72 hours, 250 grams of plant leaf powder were extracted with 80 percent methanol (1,5 liter) using soxhlet equipment.Under reduced pressure, the extract solution was concentrated to dryness in a rotary evaporator to give dried crude extract, which was frozen at -20°C until used to prepare the needed concentrations.

Plant flavonoid separation

Chena (12) Methanolic extract of *Ficus religiosa*. *The* soxhlet apparatus was used to extract 60 grams of plant leaf powder with 100 percent methanol (600 ml) for 72 hours at 60 °C The extract solution was dried using a rotary evaporator to the point where it could be frozen at -20 °C until it was utilized to prepare the required concentrations.

High performance liquid chromatography (HPLC)

The effective compounds flavonoids were analyzed using high performance liquid chromatography HPLC under various conditions. HPLC (Shimadzu 10 AV-LC) flavonoid chromatogram Separation column C18-DB, 3m particle size (50 mm * 2.0 mm I.D), The detector wavelength was 285 nm. The mobile phase gradient was 0.05 percent trifluoroacetic acid in deionized water, and the solvent B was 0.05 percent trifluoroacetic acid in methanol, PH 2.5, with a flow rate of 1.1 ml/min. To do the test, the flavonoid was mixed with methanol alcohol until it was at a concentration of 40 g.

Table (1): Preparation of stock solutions of plant active compound

Plant active compound	Dose	Amount of solvent	Primary concentration
Crude extract	500 mg	10 ml (D.W)	50 mg /ml = 50000 µg /ml
Flavonoid comopound	500 mg	10 ml (D.W)	50 mg /ml = 50000 µg /ml

Antibiotic solutions

Stock solution preparation (13). The antibiotic stock was prepared using the solution described in the table (2).

Table (2): Preparation stock solution of antibiotic

Antibiotics	Dose	Quantity of solvent	Concentration Primary
Ciprofloxacin	500 mg	10 ml (D.W)	50 mg /ml =50000 µg /ml

Detection of bacterial capacity for slime layer formation and biofilm formation

The ability of *E. coli* to produce slime layers and form biofilms was determined using two methods: Congo red agar (CRA) and 96-well microtiter plates (MTP). The ability 50 *E. coli* isolates to produce a slime layer was evaluated.

Congo red agar (CRA) As follows, the medium was preparing

Brain Heart agar.....37 g/L
NACL. ……..15 g/L
Sucrose 38g/L
Red Congo 0.8g/L

All of these materials were dissolved in one liter of distilled water and then autoclaved. The Congo red stain was dissolved in 100 ml of D.W. and autoclaved separately at 121C/1.5 pound for 15 minutes, then added to the remaining ingredients once the agar had cooled to 55°C and been poured into sterile Petri dishes.

Biofilm production determination

By modifying the Crystal Violet (CV) test the production of biofilms was evaluated semi-quantitatively. The CV assay is based on the fact that the dye penetrates and binds to extracellular molecules that are negatively charged, like cell surface molecules and polysaccharides in mature biofilm extracellular matrix. This gives information about how many cells are attached to the biofilm Columns 1-11 were filled with180 µL of tryptic soy broth and 20 µl of bacteria suspension. Column 12 was filled with TSB broth alone and used as a negative control. At 37°C, incubate the microtiter plate for 24 hours Following incubation, the media were gently removed from the microtiter plate using inversion and the wells were gently washed with phosphate buffer saline (PBS) or sterile distilled water For 15 minutes, the cells adhered to the microtiter plate were stained with 150 µl of crystal violet solution 2% The dye was removed with PBS and the microtiter plates were dried at 40°C for 15 minutes The biofilm was quantified by adding 150 µl of 70% acetic acid to each well and measuring the optical density (OD) at 595 nm using an Elisa reader. The experiment was done three times, and the mean optical density (OD) value was use (14).

Molecular assay

Extraction of genomic DNA

DNA was extracted from *E. coli* isolates using a commercial extraction kit (PrestoTM Mini gDNA Bacteria Kit Quick Protocol, Geneaid,), following the manufacturer's instructions, for DNA purification from gram negative and gram-positive bacteria as follows:

Primer selection in this study

Forward and reverse primer sequences and PCR products that detect genes *fimH* and *pap* in*E.coli* were chosen in this study as shown in Tables (3)

Table (3): Primers sequences used in *E. coli* bacteria

Name of primers	Primer sequenc	Size products
***Pap*–F**	GCAACAGCAACGCTGGTTGCATCAT	336
***Pap*–R**	AGAGAGAGCCACTCTTATACGGACA	336
***fimH*–F**	GAGAAGAGGTTTGATTTAACTTATTG	560
fimH–R	AGAGCCGCTGTAGAACTGAGG	560

PCR amplification

PCR reaction tubes were placed in a thermo-cycler PCR instrument, DNA amplified as in the conditions indicated in Table (4) for monoplex PCR of each gene. The temperature and time of PCR program optimized by using gradient PCR

Table (4): Program condition of monoplex PCR amplification for each gene in this study

Gene	Step	No. of cycle	Temperature	Time (m:s)
FimH	Initial denaturation	1	94 ºC	4 min
	Denaturation	35	94 ºC	30 second
	Annealing		58 ºC	1 min
	Extension		72 ºC	1 min
	Final extension	1	72 ºC	5 min
Pap	Initial denaturation	1	94 ºC	5 min
	Denaturation	35	94 ºC	30 second
	Annealing		60 ºC	1 min
	Extension		72 ºC	1 min
	Final extension	1	72 ºC	5 min

Antibiotic biofilm inhibition assay

He assay was performed similarly to the method that was described by (15). The antibiotic used in this assay were Ciprofloxacin. Transferring 3-5 colonies into a tube containing 5ml of normal saline (NaCl 0.85 %), and then the wells of the microtiter plate are filled with 100µl of MHB. Thereafter, a 20 µl quantity of previously prepared bacterial suspension is added to each well. then 100 µl of antibiotic, ciprofloxacin. The negative control wells contained only broth (200 µl of MHB) were then incubated for 24 hrs. Following incubation, the wells were rinsed twice with 300 ml of phosphate buffer saline. The stain was washed with (DW) or (PBS) and then re suspended in each well for 150 µl of acetic acid 70%. densities of stained adherent bacteria were determined with a micro-ELISA reader at a wavelength of 595nm (13).

Biofilm inhibition assay by active plant source

This method was carried out according to (16) . with a modification of the used media. *E. coli* from fresh agar were cultured on 96 well U-bottom tissue culture plates that were filled with 100µl of MHB. and added 20 µl of bacterial suspension. Thereafter, add 100 µl plant extract for serial dilution. The negative control wells contained only broth (200 µl of MHB) and were then incubated for 24 hrs. Following incubation, the wells were rinsed twice with 300 ml of phosphate

buffer saline. The stain was washed with (DW) or (PBS) and re suspended in each well for 150 µl of 70% acetic acid. of stained adherent bacteria were determined with a micro-ELISA reader at a wavelength of 595nm(16).

Checkerboard microtiter assay estimation of synergism effect between active plant material and antibiotic

The checkerboard assay method was used to study the synergy between the crude with ciprofloxacin and flavonoid compound with ciprofloxacin, for *E. coli*(17). The first step is to add 100µl of sterile Muller Hinton broth to all wells, and the second step is to prepare four concentrations of plant extracts (128, 64, 32, and 16 µg) with four concentrations of antibiotics (1,2,4, and 8 µg). The process of multiplying the first antibiotic with a concentration on the first plant, the first antibiotic with a concentration on the second plant, the first antibiotic with a third concentration plant, and the first antibody with a fourth concentration plant. Column 11 contained just broth and bacterial inoculum as a positive control, while column 12 contained just media as a negative control. A 20 l bacterial inoculum (1 x 106 cfu/ml) was added to all wells except (columns 12), and well mixed. All plates were incubated over night at 37°C in aerobic conditions. After incubation, the plates were scanned at a wavelength of 595 nm using an ELASA reader to observe growth in wells. Using the formula FIC = MIC of antibiotic in combination or MIC of antibiotic alone, in vitro interactions between active plant chemicals and antimicrobial drugs were determined and measured. FIC of active plant compound = MIC of active plant extract in combination or MIC of plant extract FICI = FIC of an antibiotic + FIC of a plant extract;the FIC index (FICI) was as follows.

FICI ≤0.5 means synergy.
FICI > 0.5–1 means additive.
FICI 1–4 means indifference.
FICI > 4 means antagonism.

Statistical analysis

Is performed Statistical Package for the Social Sciences (SPSS) (version 25). Chi-square ($\chi 2$) test used for independent and goodness of fit, $P \leq 0.05$ was considered statistically significant and $P \leq 0.05$ considered High statistically significant.

Results

Identification of *E. coli*

The morphological features on culture media, biochemical tests, and the VITEK 2 compact system were used to identify *E. coli*.

Cultural characteristics of *E. coli*

A total of 119 samples were identified depending on morphological examination and cultural media. Morphologically, the collected samples were cultured on MacConkey agar and Eosin Methyl Blue (EMB) agar. The results revealed 76 (63.8%) allowed growth of gram-negative bacteria and non-growth of gram-positive bacteria (1,7). Where shown in figure (1-A) and (1-B).

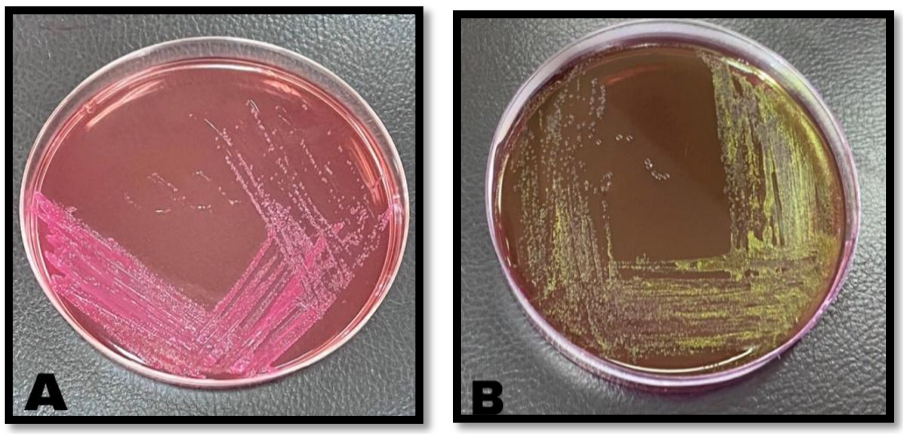

Figure (1): *E.coli* isolate on (A) MacConkey agar with lactose fermentation and pink colonies, and (B) EMB agar with a greenish metallic sheen.

Antibiotic susceptibility test (AST) of *E. Coli*

60 isolates went through the susceptibility test, for nine different of antibiotics by the disc diffusion clinical and laboratory standards institute recommended technique (CLSI, 2020) guidelines the results shown in Table (5) that results in figure showed varied levels of resistance, The study indicates the presence of highly significant difference,** (P≤0.05).

Table (5): The result of antibiotics susceptibility test for *E. coli* Isolates

Antibiotics	Resistance		Intermediate		Sensitive		*P-value*
	No.	%	No.	%	No.	%	
Ertapenem	9	15	3	5	48	80	<0.0001**
Nitrofurantoin	33	55	3	5	24	40	<0.0001**
Trimethoprime-Sulfanethaxole	44	73.3	4	6.7	12	20	<0.0001**
Norfloxacin	38	63.3	3	5	19	31.7	<0.0001**
Piperacilln-Tazobactam	16	26.7	2	3.3	42	70	<0.0001**
Gentamicin	30	50	4	6.7	26	43.3	<0.0001**
Amoxicillin	23	38.3	3	5	34	56.7	<0.0001**
Ciprofloxacin	24	40	1	1.7	35	58.3	<0.0001**
Ceftriaxone	35	58.3	1	1.7	24	40	<0.0001**

Data is presented as Chi-square (χ^2) goodness of fit. ** Significant at P ≤ 0.05.

The antimicrobial susceptibility of *E.coli* isolates showed 33 isolates (55%) resistant to Nitrofurantoin, 38 isolates (63.3%) resistant to Norfloxacin, and 44 isolates (73.3%) resistant to trimethoprim-sulfamethoxazole. 24 isolates (40%) resist Ciprofloxacin, 16 isolates (26.7%) resist Piperacillin tazobactam, 30 isolates (50%) resist Gentamicin, 23 isolates (38.3%) resist Amoxicillin, and 35 isolates (58.3%) resist Ceftriaxone. There was less resistance to 9 isolates (15%) to Ertapene than to other antibiotics. The highest resistance to Ceftriaxone was obtained, which is 19 mm, and the lowest resistance was to trimethoprim-sulfamethoxazole, which is zero.

Biofilm formation in *E. coli*

Congo red agar

Congo red agar method, black colonies' slime layers were observed for the biofilm production.

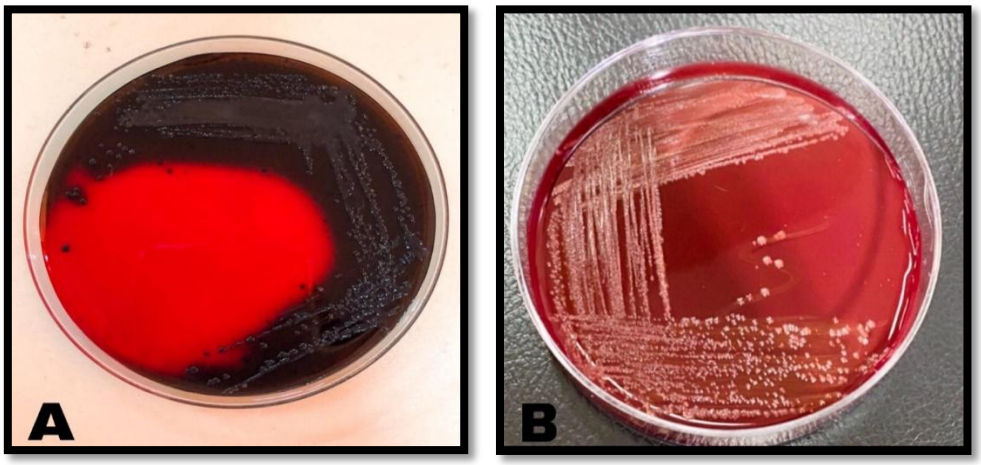

Figure (2): Biofilm formation on Congo red agar. In Figure A, black colonies show biofilm formation, while in Figure B, red colonies show non-formation biofilm.

Microtiter plate (96 wells plate)

Microtiter plate method, which was previously used for biofilm formation pinpointing, gave the following results for *E. coli*, weak = 15 (30%), moderate = 4 (8%), strong = 21 (42%), non-biofilm = 10 (20%).

Extraction and quantification of DNA concentration and purity in bacteria *E. coli*

The DNA of 30 isolates was successfully extracted. 30 of *E. coli* were extracted efficiently by the Presto TM Mini gDNA Bacteria Kit Quick Protocol (Geneaid). the concentration of the DNA extracted was in the range of (34.6–180) ng/µl with a purity range of (1.75-2.02) in bacteria E. coli. Show Figure (3) in E. coli illustrate the presence of a single band of extracted DNA, which indicates the efficiency of the method used in the extraction of DNA ransilluminator.

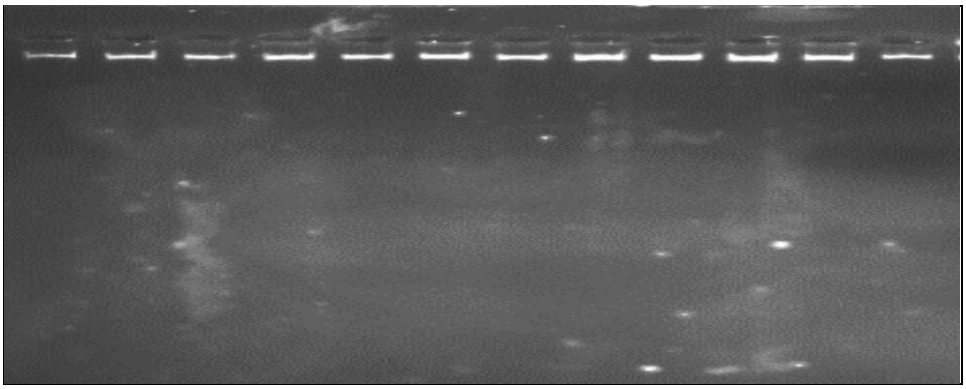

Figure (3): Gel electrophoresis of whole genome DNA of *E. coli*. Agarose 1%, 70 V for 1hrs., visualized on a UV trans illuminator.

Molecular detection of Biofilm in *E. coli*

Thirty isolates of *E. coli* bacteria that showed resistance to antibiotics and also biofilm production (strong, moderate, and weak) were selected to detect the biofilm genes pap and fimH gene, which play a major role in the formation of biofilms in *E. coli* bacteria. By controlling the production of Curli

(fimbriae) and other biofilms (6). Using PCR technology (polymerase chain reaction) and gel electrophoresis to cut DNA and using the initiator to verify the presence of fimH and pap genes in the bacterial isolates under test. The results showed that 21 (70%) affiliated bacterial isolates of E. coli possess the fimH gene and 17 (56.6%) isolates possess the pap gene.as shown Figures (4) and (5).

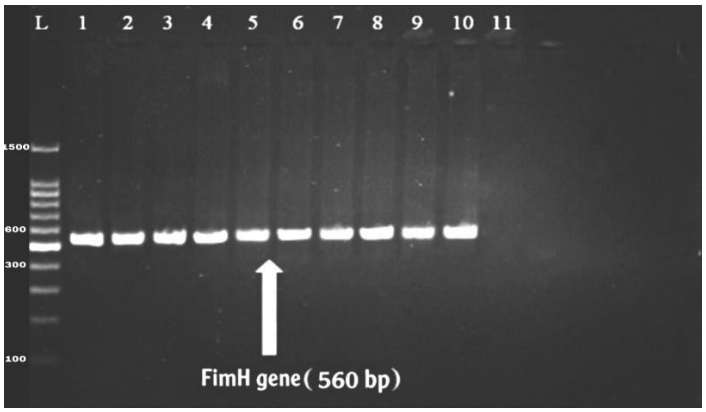

Figure (4): Gel electrophoresis of *E. coli fimH* (560bp) that was amplified by conventional PCR. Agarose at 1.5%, 70 V/cm for 90 minutes, stained with ethidium bromide dye, and viewed with an ultraviolet trans illuminator. 100 bp. DNA ladder Lane (L). Lane (11): Negative control.

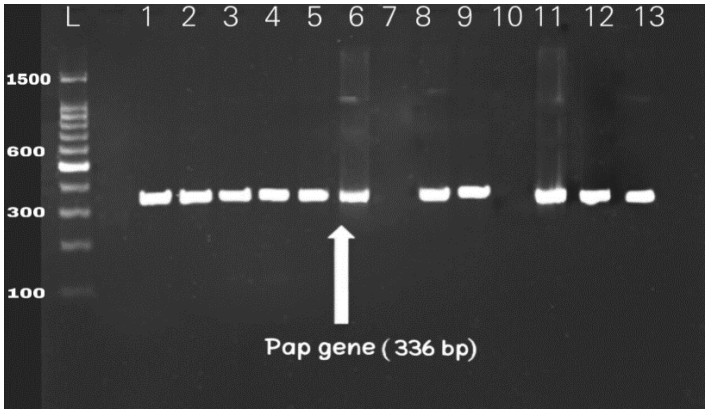

Figure (5): Gel electrophoresis of *E. coli pap* (336) that was amplified by conventional PCR. Agarose at 1.5%, 70 V/cm for 90 minutes, stained with ethidium bromide dye, and viewed with an ultraviolet trans illuminator. 100 bp DNA. ladder Lane (L). Lane (10,7): Negative control.

Crude extract and total flavonoid separation of *F. religiosa*

Methanol was chosen as the extraction solvent in the soxhlet system since it had a high extraction capacity and excellent polarity (18) agreed with this outcome for the extraction of 80% methanol. Furthermore, the polarity of the water is strong, but not all plant material can be dissolved and extracted with it. As a result, 80% of methanol is made up of methanol and water for high extraction capacity. The result of the extraction extraction result for crude and total flavonoid of *Ficus religosa* was 250 and 60 gm, respectively, and the yield extract was 28 and 12 gm, respectively. Crude and flavonoid extract concentrations reached 11.2% and 20%, respectively. This ratio is estimated by the equation where the extraction ratio (ER) was determined based on (20). using the following equation:

ER = (extract concentration (yield) (g)/fresh plant initial weight (g)) x 100.

Analysis of total flavonoid by HPLC technique for *Ficus religosa*

The high-performance liquid chromatography (HPLC) technique was also used for the characterization of plant-isolated flavonoid samples and comparing them with their standards according to(21,22). As depicted in Figure (6), this characterization revealed the presence of five peaks in the flavonoid-separated *F. religosa* sample. The first, second, third, and fourth peaks occurred at retention times of 5.79, 18.02, 24.15, 30.08, and 35.58 min, respectively. The appearance of fifth peaks of partially purified flavonoids refers to a good separation of the flavonoids. On the other hand, the presence of some smaller peaks of separate flavonoids refers to the presence of secondary compounds derived from the main flavonoids and also indicates the efficiency of the extraction method used when compared with various techniques for the extraction of *Ficus religosa* (23).

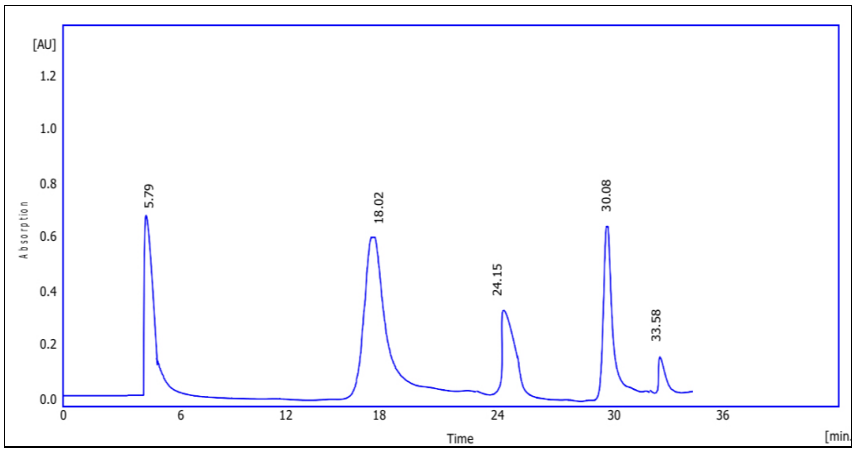

Figure (6): HPLC for *ficus religosa* total flavonoid extract.

Effect of antibiotic alone on biofilm formation

The microtiter plate method, which was used for antibiotics alone, affected biofilm formation. Figure (7) shows the effect of the antibiotic alone on biofilm formation for *E. coli*. The best biofilm formation inhibitor for CIP in concentration (128 µg/mL) was 83.3%.

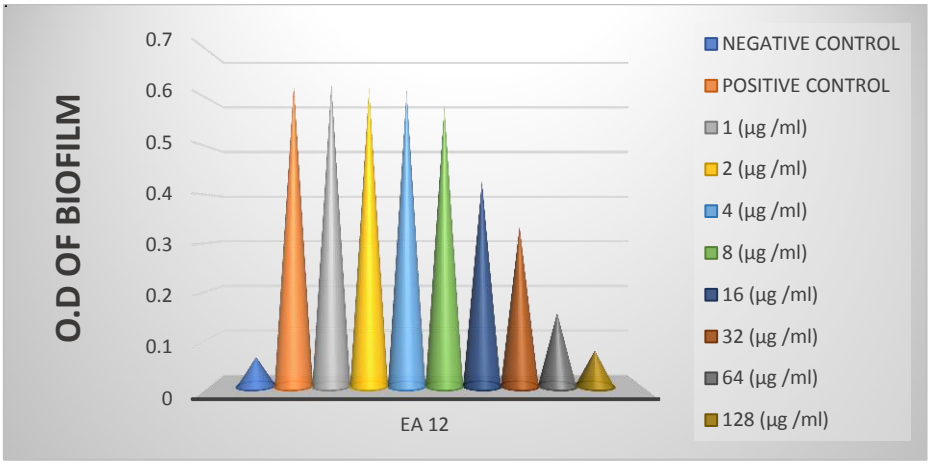

Figure (7): Ciprofloxacin alone against *E. coli* biofilm formation.

Effect of plant extract alone on biofilm formation

The current study measured biofilm formation by *E. coli* in the response to MICs of crude extract and flavonoid by using microtiter plate assay and the crystal violet staining method. The responses were quantified by measuring the absorbance of stained biofilms at 590 nm with an ELISA reader. The results showed that crude extract and flavonoid compounds have antibiofilm activity against bacteria *E. coli*. The inhibition of biofilm formation was 90.6% for bacteria E. coli when using crude extract at 512 ug/ml concentration. While the inhibition of biofilm formation was 80.6% for *E. coli* bacteria when using flavonoid compound at 512 ug/ml concentration. The effect of plant extract alone on biofilm formation was shown in figures (8), and (9).

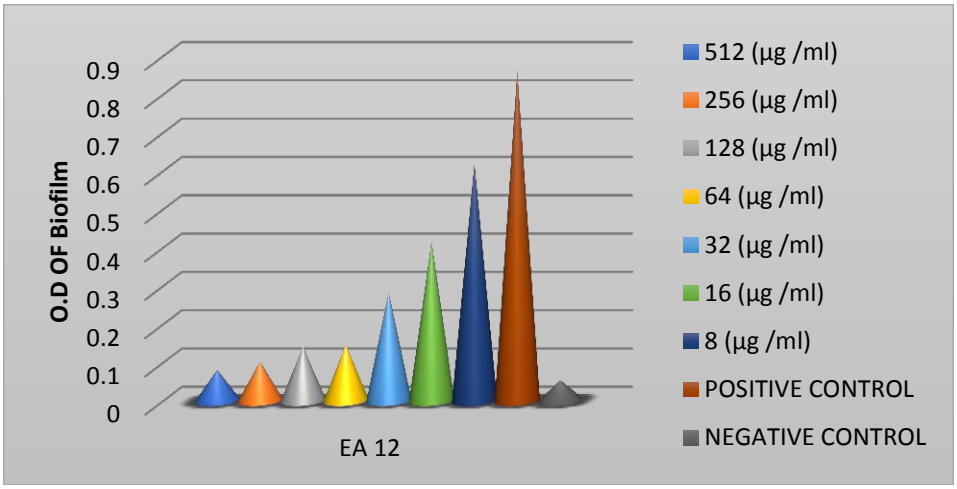

Figure (8): Crude extract alone against *E. coli* biofilm formation.

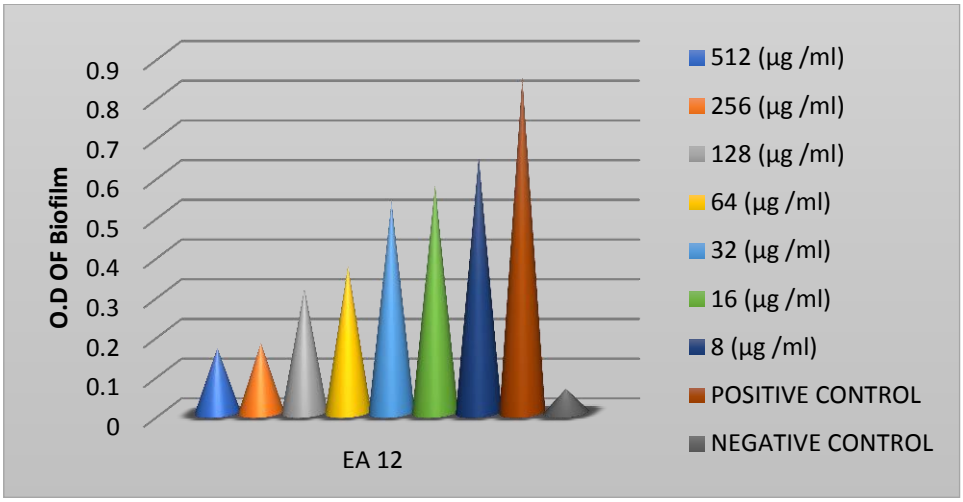

Figure (9): Flavonoid compound alone against *E. coli* biofilm formation.

Effect of antibiotics and plant extract combinations on biofilm formation

The microtiter plate method, which was used for antibiotics, with the effect of the composition of plant extracts on the formation of biofilms. The results are shown for *E. coli* are shown in figures(10) and (11), where the combination of antibiotic (CIP) with crude extract and flavonoid compound was successful.

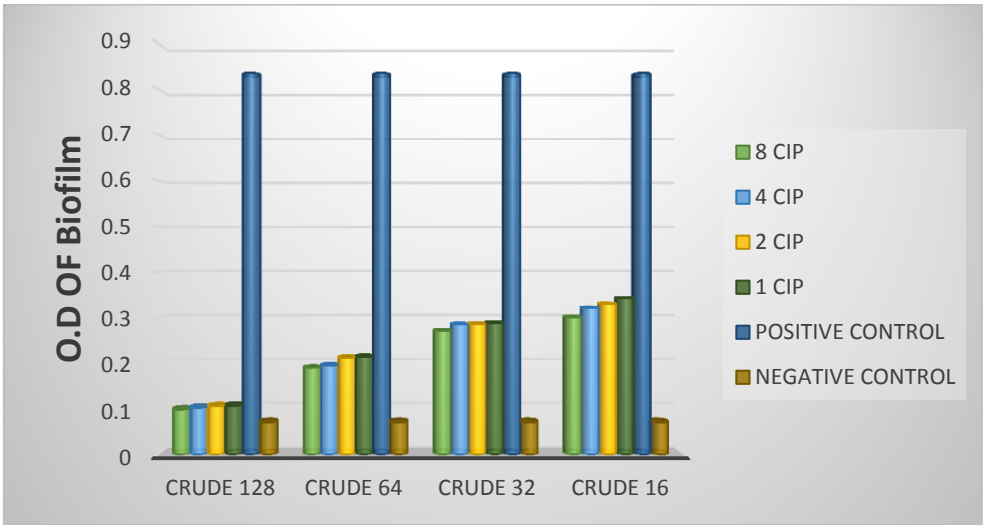

Figure (10): Combination of Ciprofloxacin and crude extract against biofilm formation of *E. coli.*

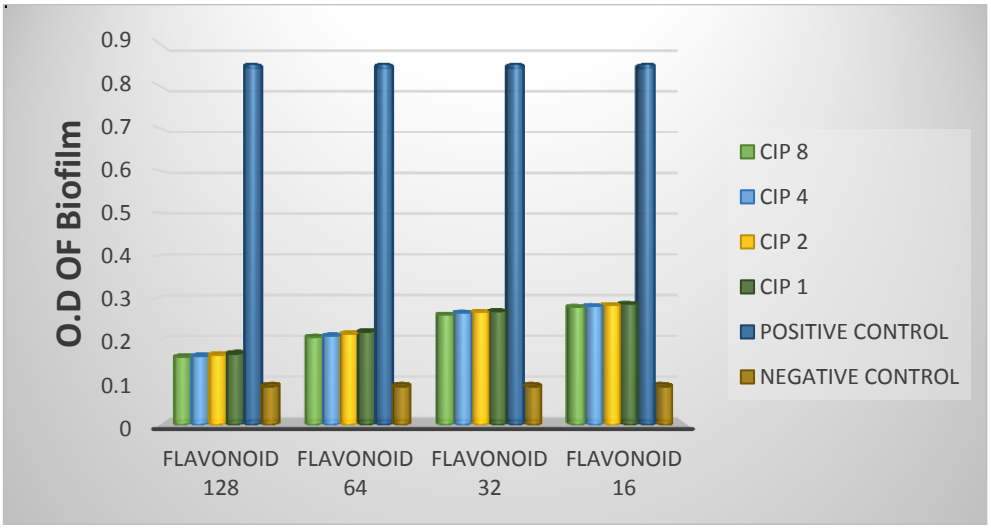

Figure (11): Combination of Ciprofloxacin and flavonoid compound against biofilm formation of *E. coli*.

Discussion

The biofilm-associated infections have been well suited to a confrontation for doctors because of the insistence of biofilms that act of going up multidrug resistance. CIP is known for their effects as biofilm distraction antibiotic antimicrobial such destruction of biofilm, alteration of the bacterial outer membrane, and inhibition of expression of virulence factors (19). CIP are inhibition the cell wall synthesis by effect on binding of penicillin-binding proteins (PBPs). CIP are made away through a porin in the across of outer membrane of *E. coli*, allows selective penetration of the drug with basic amino acids. The current study suggests a useful outcome combination therapy of the CIP with crude extract and Flavonoid compound in urine and stool infected by *E. coli* that is remarkable in biofilm forming inhibition. The majority of biofilm-related chronic illnesses are caused by the persistence of polymicrobial biofilms, which is not taken into account by conventional and contemporary anti-biofilm therapy. Thus, there is no ideal approach to completely removing biofilm, but the key would be the simultaneous application of agents employing mechanisms with synergistic potential in order to disrupt the structure of biofilm and kill bacteria. Using computational methods to comprehend anti-biofilm mechanisms thoroughly seems necessary. They could be used to study how anti-biofilm compounds work, especially to find out how well they work and how they might affect the development of new types of resistant microorganisms. This study concludes that synergistic activity of crude extract and flavonoid compound with commercial antimicrobials CIP showed promising results. CIP with combination seems to be profitable of biofilm treatment generated from *E. coli* in contrast expressing the antibiotics only. To perfect of our knowledge, this report is citing first in-vitro state combination therapy of the CIP+crude extract and CIP+ Flavonoid compound. The CIP, crude, and flavonoid vitro results promising to choose the combination efficacy therapy. The combination of CIP (8 ug/mL) also crude and flavonoid (128 ug/mL) resulted appear synergism in

Chequerboard assay against planktonic cells (FICI < 0.5), depending on the result for CIP inhibition biofilm alone was(16ug/ml) and become (1,2,4, and 8 ug/ml) when combined. Combination at inhibitory concentrations also appears benefit capacity to biofilm formation inhibition after 24 h.

Conclusion

Most of the studies for crude extract, flavonoid compound, and CIP revealed an ability of inhibiting antibacterial activities. In addition, such a synergistic combination of crude extract and flavonoid compound with CIP in clinical situations is especially desired, thus in addition potency of the CIP with crude extract and flavonoid compound joining in the treatment of biofilm can also be exploited. The study explained the alone and combinatorial impact of CIP and crude extract and flavonoid compound in vitro generated *E. Coli*.

Reference

1. Foster, Sr., R. T. (2008). Uncomplicated urinary tract infections in women. Obstetrics and Gynecology Clinics of North America, 35(2): 235-248.
2. Kolawole, A. S.; Kolawole, O. M.; Kandaki-Olukemi, Y. T.; Babatunde, S. K.; Durowade, K. A. and Kolawole, C. F. (2010). Prevalence of urinary tract infections (UTI) among patients attending Dalhatu Araf Specialist Hospital, Lafia, Nasarawa state, Nigeria. International Journal of Medicine and Medical Sciences, 1(5): 163-167.
3. Okonko, I. O.; Ijandipe, L. A.; Ilusanya, O. A.; Donbraye-Emmanuel, O. B.; Ejembi, J.; Udeze, A. O., *et al*. (2009). Incidence of urinary tract infection (UTI) among pregnant women in Ibadan, South-Western Nigeria. African Journal of Biotechnology, 8(23).
4. Kawamura-Sato, K.; Yoshida, R.; Shibayama, K. and Ohta, M. (2010). Virulence genes, quinolone and fluoroquinolone resistance, and phylogenetic background of uropathogenic *Escherichia coli* strains isolated in Japan. Japanese Journal of Infectious Diseases, 63(2): 113-115.
5. Liu, Y.; Yan, X.; Deb Roy, C.; Fratamico, P.M.; Needleman, D.S.; Li, R.W., *et al*. (2015) *Escherichia coli* O- Antigen Gene Clusters of Serogroups O62, O68, O131, O140, O142, and O163: DNA Sequences and Similarity between O62 and O68, and PCR-Based Sero grouping. Biosensors (Basel), 5(1): 51–68.
6. Ogasawara, H.; Yamamoto, K. and Ishihama, A. (2010). Regulatory role of MlrA in transcription activation of csgD, the master regulator of biofilm formation in *Escherichia coli*. FEMS Microbiology Letters, 312(2): 160-168.
7. Davies**,** J. and Davies, D. (2010). Origins and evolution of antibiotic resistance. Microbiol Microbiology and molecular biology Reviews, 74(3):417-433.
8. Sarowska, J.; Futoma-Koloch, B.; Jama-Kmiecik, A.; Frej-Madrzak, M.; Ksiazczyk, M.; Bugla-Ploskonska, G., *et al*. (2019). Virulence factors, prevalence and potential transmission of extraintestinal pathogenic Escherichia coli isolated from different sources: recent reports. Gut Pathogens, 11(1):10.
9. Lüthje, P. and Brauner, A. (2014). Virulence factors of uropathogenic *E. coli* and their interaction with the host. In: Advances in Microbial Physiology: Elsevier. 65: 337-372.
10. Borges, A.J.; Saavedra, M. and Simoes.; M. (2015). Insights on antimicrobial resistance, biofilms and the use of phytochemicals as new antimicrobial agents. Current Medicinal Chemistry, 22(21): 2590-2614.
11. Upadhyay, A.; Upadhyaya, I.; Kollanoor-Johny, A. and Venkitanarayanan, K. (2014) Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. BioMed research international, 2014, 761741.
12. Fua, W.; Chena, J.; Caia, Y.; Leia, Y.; Chenb. L.; Peic L., *et al*. (2010). Antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective potential of the extract from Parathelypteris nipponica (Franch. et Sav.) Ching. Journal of Ethnopharmacology, 130(3): 521–528.
13. Wiegand, I.; Hilpert, K. and Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory

concentration (MIC) of antimicrobial substances. Nature protocols, 3(2): 163-175.
14. Mathur, T.; Singhal, S.; Khan, S.; Upadhyay, D. J.; Fatma, T. and Rattan, A. (2006). Detection of biofilm formation among the clinical isolates of *staphylococci*: an evaluation of three different screening methods. Indian Journal of Medical Microbiology, 24(1): 25-29.
15. Mataraci, E. and Dosler, S. (2012). In vitro activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin resistance *Staphylococcus aureus* biofilms. Antimicrobial agents and chemotherapy, 56(12): 6366–6371.
16. Sandasi, M.; Leonard, C. M. and Viljoen, A. M. (2010). The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocyto* genes. Letters in Applied Microbiology, 50(1): 30-35.
17. Sopirala, M. M.; Mangino, J. E.; Gebreyes, W. A.; Biller, B.; Bannerman, T.; Balada-Llasat, J. M. *et al*. (2010). Synergy testing by Etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant *Acinetobacter baumannii*. Antimicrobial agents and chemotherapy, 54(11): 4678-4683.
18. Raul, G. B.; Andres, F. O.; Irma, F. and Francisco, M. (2003). Electrical conductivity of hydrophobic ally modified polyelectrolytes in methanol /water solution. *Journal of the* Chilean Chemical Society. 48(1): 717- 970.
19. Nanda kumar, V.; Chittaranjan, S.; Kurian, V.M. and Doble, M. (2013). Characteristics of bacterial biofilm associated with implant material in clinical practice. Polym Journal, 45(2): 137–152.
20. Al-Shahat, N.A. (1986). Plants and Medicinal Herbs. Dar Al-Behaar, Beirut. 140- 146.
21. Srivastava, J. K. and Gupta, S. (2009). Extraction, characterization, stability and biological activity of flavonoids isolated from chamomile flowers. Molecular and cellular Pharmacology 1(3): 138.
22. Obouayeba, A. P.; Djyh, N. B.; Diabate, S.; Djaman, A. J.; N'guessan, J. D.; Kone, M., *et al*. (2014). Phytochemical and antioxidant activity of Roselle (Hibiscus sabdariffa L.) petal extracts. Research Journal of pharmaceutical, Biological and Chemical Sciences, 5(2): 1453–1465.
23. Molnar, M.; Mendešević, N.; Šubarić, D.; anjari, I. and Jokić, S. (2017). Comparison of various techniques for the extraction of umbelliferone and herniarin in Matricaria chamomilla processing fractions. Chemistry Central Journal, 11(1): 1–8.
24. AL-Shuwaikh, A. M., Ibrahim, I. A., & Al-Shwaikh, R. M. (2015). Detection of E. coli and rotavirus in diarrhea among children under five years old. Iraqi Journal of Biotechnology, 14(1).