Scholars Academic Journal of Biosciences
Abbreviated Key Title: Sch Acad J Biosci
ISSN 2347-9515 (Print) | ISSN 2321-6883 (Online)
Journal homepage: https://saspublishers.com

OPEN ACCESS

Microbiology

Investigation of Synergistic Effect of Silver Nanoparticles Using Yogurt Lactobacillus Flora on Escherichia Coli with Multiple Drug Resistance

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DOI: 10.36347/sajb.2022.v10i05.001 | **Received:** 23.03.2022 | **Accepted:** 27.04.2022 | **Published:** 07.05.2022

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Abstract **Original Research Article**

Background and Objective: Currently, many valuable drugs have lost their impact on Escherichia coli, and Escherichia coli drug resistance is the major reason for the treatment failure of nosocomial infections caused by this bacterium. This study aims to investigate the effect of silver nanoparticles using yogurt Lactobacillus flora on Escherichia coli with multiple drug resistance. ***Materials and Methods:*** This cross-sectional-experimental study was conducted in 2019 on Escherichia coli strain from some clinical centers in Ardabil, Iran. The isolates were performed based on approved biochemical tests to identify Escherichia coli bacteria. Antibiotic susceptibility testing was carried out by diffusion method. Then, its physical and chemical properties were determined by spectrophotometry (UV), SEM microscopy, and FESEM. Then, the synergistic effect of silver nitrate growth inhibitor was investigated using yogurt Lactobacillus flora by disk diffusion method according to standard (CLSI). ***Findings:*** In the present study, out of 100 samples, 37 strains of Escherichia coli were positive. The highest resistance of Escherichia coli isolates was to ciprofloxacin with 87% and the highest susceptibility was to nitrofurantoin with 76%. The size of silver nanoparticles according to the image of TEM and FESEM was both shown to be 16 nm in diameter. Silver nitrate using yogurt lactobacilli flora was able to reduce the combination of Escherichia coli strains with antibiotics, especially ciprofloxacin. ***Conclusion:*** The results of the present study showed that silver nanoparticles synthesized using yogurt lactobacilli flora with a diameter of 16 nm have antimicrobial activity against Escherichia coli. Considering the effectiveness of silver nanoparticles using yogurt Lactobacillus flora on ciprofloxacin-positive Escherichia coli, it can be used as a candidate drug against Escherichia coli in the future.

Keywords: Escherichia coli, Multiple drug resistance, Silver nanoparticles, Prebiotic.

INTRODUCTION

Escherichia coli is regarded as the most typical cause of urinary tract infections, which accounts for about 90% of urinary tract infections in young women. Urinary tract infections are more common in women than in men, so that half of women have experienced a urinary tract infection at least once in their lifetime. According to studies conducted in different communities, gram-negative bacilli have been reported as the most common etiological factor in infants, men, women, pregnant women and the elderly, patients with spinal cord injuries and following the use of urinary catheters and patients with diabetes, and immunocompromised patient. Escherichia coli is a gram-negative bacillus of the Enterobacteriaceae family that causes various human infections such as septicemia, inflammation of the stomach and intestines, neonatal meningitis, gallbladder and bile duct infections, wound infection, pneumonia and peritonitis (peritoneal infection) and especially urinary tract infections and kidney failure [1].

The emergence of antibiotic-resistant strains in the treatment of infections caused by this organism, especially when only one type of antibiotic is used, is not unexpected and is a concern for clinicians. Considering the increased resistance of bacteria to antibiotics, it is necessary to conduct a study to identify and use other compounds and formulas as an alternative to antibiotics or adjuvants with them in the treatment of infections caused by various bacteria such as Escherichia coli [2-4]. Today, the use of metals such as silver, copper, gold, aluminum in various forms, including nanoparticles as antimicrobial compounds has become popular [5]. The medicinal and therapeutic properties of silver have been known since 2000 years ago. Beginning in the 19th century, silver compounds were used in antimicrobial equipment [6]. Today, this metal is used as an antibacterial compound in various forms [7]. Numerous reports have shown that silver nanoparticles have antimicrobial effects, so that by changing the bacterial membrane morphology, it increases the permeability of silver nanoparticles and uncontrollable penetration of silver nanoparticles occurs into the cell and leads to cell death [8].

In general, there are various physical and chemical methods such as chemical reduction, photochemical reduction, ultraviolet radiation, and micro-laser for the synthesis of silver nanoparticles. Chemical methods do not have good efficiency due to the production of toxic chemical compounds, and compared to other methods, environmentally friendly bio-methods are preferred for the synthesis of silver nanoparticles. As these methods are one-step and do not require reduction and stabilizing compounds. Among bio-methods, prebiotics and plant species are a suitable option for the synthesis of silver nanoparticles. The method of synthesis by prebiotics has low cost and high efficiency and leads to the production of nanoparticle crystal structures of different sizes and this depends on the nature of prebiotics, pH, temperature and incubation time [9].

Compared to other metals, silver nanoparticles have a high toxicity on microorganisms. These particles can also be used successfully in the treatment of infections as health and pharmaceutical agents. In addition to effective antibacterial properties, silver nanoparticles have antiviral, antifungal, and anti-inflammatory effects [2]. This study aims to investigate the effect of silver nanoparticles using yogurt Lactobacillus flora on Escherichia coli with multiple drug resistance.

MATERIALS AND METHODS

Initial phenotypic identification of bacteria

The present cross-sectional-experimental study was conducted in four months on those who went to some clinical centers in Ardabil with symptoms of urinary tract infections such as burning and frequent urination and were referred to the laboratory for examination at the diagnosis of the treating physician. Been approved in Ardabil Azad University with the code 162359366. Samples were cultured on selective EMB agar medium and incubated at 37 ° C for 24 hours.

The presence of metallic green colonies indicates the presence of Escherichia coli (Figure 1).

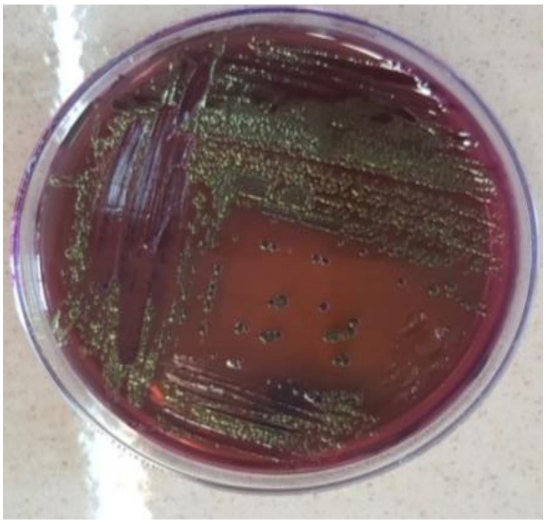


Figure 1: Production of green metallic sheen by Escherichia coli

After observing metallic sheen in EMB culture medium, bacterial colonies were examined by biochemical tests of SIM, TSI, EMB, MR-VP, Simon Citrate.

Disk diffusion method

Antibiotic susceptibility was performed by disk diffusion method according to CLSI instructions. Disks of ciprofloxacin (5 micrograms), gentamicin (10 micrograms), nitrofurantoin (10 micrograms), amikacin (30 micrograms), penicillin (1 microgram), cotrimoxazole (10 micrograms), nalidixic acid (15 micrograms), cefotaxime (30 micrograms), and cefazolin (30 micrograms) were performed.

Preparation of solutions of silver nanoparticles

First, local milks of Ardabil city were collected under sterile conditions, then yogurt was prepared. The prepared yogurt was left at room temperature for 72 hours to become sour. 5 g of local yogurt was mixed with 100 cc of distilled water and centrifuged for 15 minutes. The aqueous extract of the resulting lactobacilli was kept at 4° C for subsequent experiments. Then, 10 cc of the extract enriched with the prepared yogurt Lactobacillus flora was mixed with 90 cc of 1 mM silver nitrate solution and the solution was placed on a magnetic stirrer at room temperature for 24 hours. The solution containing the nanoparticles was then centrifuged at 12000 rpm for 15 minutes, then the supernatant was discarded. In order to observe the color changes on the absorption of the solution, the silver was examined in the range of 450-700 nm using a spectrophotometer.

Determining the size of nanoparticles and morphology

Silver nanoparticles synthesized from extract enriched with yogurt Lactobacillus were analyzed and evaluated for determination of size, structural properties, optical properties, and morphology by X-ray diffraction (XRD), field emission scanning electron microscope (FESEM) and transmission electron microscopy (TEM-SEM), respectively. To determine the crystalline phases of the synthesized silver nanoparticles, also to measure the crystalline constants of silver nanoparticles and to calculate the size of the crystals in the X-ray diffraction pattern of the samples were used.

In order to determine the size and dispersion distribution of silver nanoparticles synthesized from yogurt extract, transmission electron microscopy with an applied voltage of 120 KV was used to emit electron beams. The morphology of the silver nanoparticles synthesized from the extract was investigated by the field emission scanning electron microscope.

Investigation of the synergistic effect of silver nitrate growth inhibitor using yogurt Lactobacillus by disk diffusion method

In this method, a suspension of 0.5 McFarland was prepared from bacteria grown in EMB agar medium. It was then cultured in three directions by sterile swap in Mueller-Hinton agar medium, and after ten minutes, the antibiotic disks were placed alone and the antibiotic disks inoculated with 250 µg/ml silver nitrate were placed in the other plate. The cultured bacteria were incubated at 35 °C for 16-20 hours. Then, the diameter of the growth inhibition zone of antibiotics alone and along with nanoparticles was measured and recorded.

FINDINGS

In the present study, in the first 4 months of (2019), out of 100 patients in some clinical centers in Ardabil, 39 urine samples after culture on EMB medium were positive colonies, which were confirmed as Escherichia coli after biochemical tests.

The results of Escherichia coli isolated antibiogram were determined relative to the taken antibiotics according to Table 2. Escherichia coli isolates were most resistant to ciprofloxacin (87%) and most sensitive to nitrofurantoin (76%).

Table 4: Antibiotic susceptibility pattern of isolated Escherichia coli strains

Antibiotic	Drug concentration	Number of resistant strains	Number of intermediate strains	Number of susceptible strains
		Percentage (frequency)	Percentage (frequency)	Percentage (frequency)
Amikacin	30 µg	71 (71)	5 (5)	24 (24)
Cotrimoxazole	10 µg	69 (69)	-	31 (31)
Gentamicin	10 µg	53 (53)	-	47 (47)
Nitrofurantoin	10 µg	21 (21)	3 (3)	76 (76)
Nalidixic acid	15 µg	60 (60)	11 (11)	29 (29)
Penicillin	1 µg	75 (75)	18	7 (7)
Ceftizoxime	30 µg	52 (52)	22 (22)	26 (26)
Cefazolin	30 µg	60 (60)	26 (26)	14 (14)
Ciprofloxacin	5 µg	87 (87)	-	13 (13)
Cefotaxime	30 µg	76 (76)	13 (13)	10 (10)

Results of analysis of silver nanoparticles using yogurt Lactobacillus flora

Figure (2)-(a) shows the TEM transmission electron microscopy of silver nanoparticles synthesized from the extract of yogurt enriched with Lactobacillus flora. As can be seen from the images, the silver nanoparticles in the picture are darker and have a spherical shape and have a suitable distribution that is related to the solvent. Because the density-solvent is less than the density of silver nanoparticles against the passage of light, and therefore silver nanoparticles are darker and the solvent is lighter in the image. The size of silver nanoparticles is 16 nm in diameter according to the TEM image, respectively

Figure (b) shows microscopic image of field emission scanning electron with magnification of 100 nm. The FESEM image shows the dimensions of silver nanoparticles and shows an almost spherical shape. Determining the size of nanoparticles through FESEM is not accurate because the resolution of FESEM is lower than TEM and therefore TEM analysis is used to express the average size. According to FESEM images, the cumulative size of silver nanoparticles is 16 nanometers.

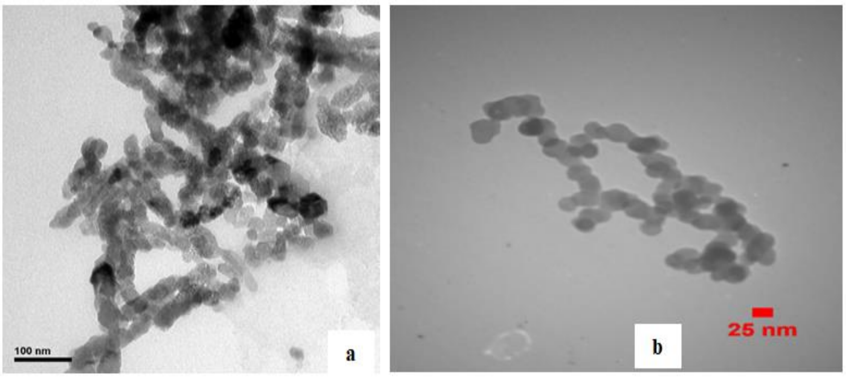
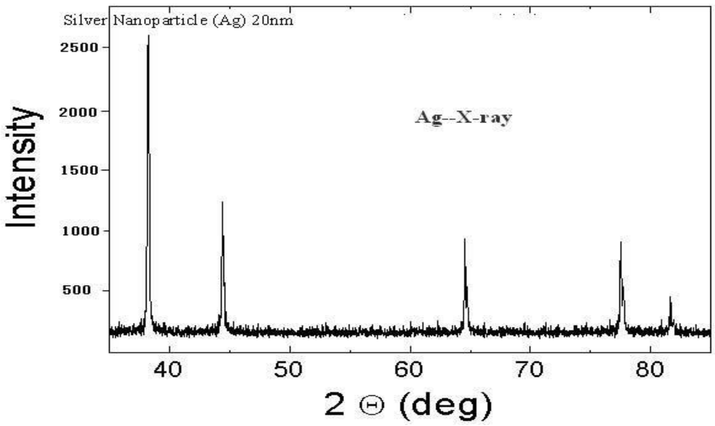


Figure 2: a) Transmission electron microscopy of silver nanoparticles synthesized from the extract of yogurt enriched with Lactobacillus flora with magnification of 25 b) microscopic image of field emission scanning electron of silver nanoparticles synthesized from the extract of yogurt enriched with Lactobacillus flora with magnification with magnification of 100

Graph 1 shows the XRD X-ray diffraction pattern of silver nanoparticles synthesized from the extract of yogurt enriched with Lactobacillus flora. As can be seen, the peaks (2700), (1300), (1000), (1000), (700) at θ=38.07°, 43.08°, 64°, 77.5°, and 86° are related to the Face Centered Cubic (FCC) structure of silver nanoparticles.

In the above study, the low diffraction pattern at half the maximum peak (2700) indicates the large crystalline size of silver nanoparticles. The crystalline size of silver nanoparticles is obtained from the Body-Scherrer equation.

In the above equation, K = 0.9 is a shape factor. λ is the wavelength of X-rays is equal to 1.6045 A°. β is the full width at half the maximum peak pf diffraction and θ is the angle corresponding to the diffraction peak. From the calculation of the following equation, the crystallize size of the nanoparticles is 16 nm.



Graph 1: XRD test of nanoparticles

Results from the synergistic effect of silver nitrate growth inhibitor using yogurt Lactobacillus flora by disk diffusion method.

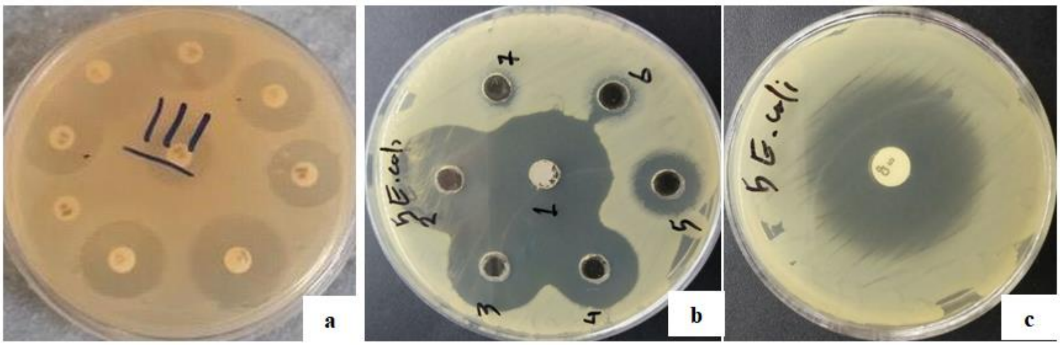


Figure (3)-a) Result of antibiogram of Escherichia coli isolate in relation to different antibiotics b) Result of antibiogram of Escherichia coli isolate with silver nitrate in relation to different antibiotics c) Result of antibiogram of Escherichia coli isolate in relation to ciprofloxacin in extract enriched with yogurt Lactobacillus flora to silver nitrate diameter of growth zone

Table 2: Diameter of the growth inhibition zone of antibiotics alone and in combination with silver nanoparticles on average diameter of the growth inhibition zone of all antibiotic disks in combination with silver nanoparticles

Type of antibiotic		Diameter of antibiotic growth inhibition zone alone (mm)	Diameter of antibiotic growth inhibition zone and silver nanoparticle (mm)
Amikacin	30 µg	71 (71)	57 (57)
Cotrimoxazole	10 µg	69 (69)	45 (45)
Gentamicin	10 µg	53 (53)	23 (23)
Nitrofurantoin	10 µg	21 (21)	20 (20)
Nalidixic acid	15 µg	60 (60)	24 (24)
Penicillin	1 µg	80 (80)	14 (14)
Cefazolin	10 (10) g	60 (60)	30 µ
Ciprofloxacin	5 µg	87 (87)	0
Cefotaxime	30 µg	76 (76)	70 (70)

DISCUSSION

Given that Escherichia coli is in the category of Gram-negative bacteria and faces more barriers to the acceptance of nanosilvers compared to Gram-positive bacteria, it responds to nanoparticles later than Gram-positive bacteria. In this study, the reason for the effects of silver nanoparticles using yogurt Lactobacillus flora on this Gram-negative bacterium despite the wall resistance can be attributed to the small diameter of silver ions and, as a result, the greater permeability of these nanoparticles. In a study, Moudg *et al*., (2006) showed that the effects of nanoparticles on cells of living organisms depend on the diameter, size, and shape of the nanoparticles [10]. Due to their small size, silver nanoparticles have a higher contact surface with the outside space and have more effects on cell membranes [11, 12]. The results of research show that the inhibitory mechanism of silver nanoparticles is due to the function of silver ions in colloidal solution. Transformation of microorganisms is also done by converting SH bonds to SA-g. In this mechanism, metallic silver nanoparticles emit silver ions over time. During the substitution reaction, these ions convert SH bands in the microorganism wall into SA-g bands, which results in the destruction of the microorganism [10, 13]. Also the innovative aspect of this study was that for the first time silver nanoparticles were synthesized using yogurt lactobacillus flora and its biological effects were studied. In general, there are chemical and physical methods for the production of nanoparticles today. A low-cost and environmentally friendly solution for the synthesis of silver nanoparticles is the use of yogurt lactobacillus flora.

In the present study, out of 100 samples, 37 (37%) Escherichia coli strains were colony positive, with the highest resistance of Escherichia coli isolates to ciprofloxacin (87%) and their highest susceptibility to nitrofurantoin (76%). The size of silver nanoparticles was shown to have a diameter of 16 nm according to the TEM and FESEM images. Silver nitrate using yogurt lactobacillus flora was able to reduce Escherichia coli strains in combination with antibiotics, especially ciprofloxacin

In the present study, out of 100 samples, 37 (37%) Escherichia coli strains were colony positive, with the highest resistance of Escherichia coli isolates to ciprofloxacin (87%) and their highest susceptibility to nitrofurantoin (76%). In the study conducted by Zolnouri *et al.*, in 2017, they reported the highest resistance to antibiotics penicillin (58.5%) and ciprofloxacin (34.66%) [14]. In a Nigerian study in 2004, all strains of Escherichia coli were resistant to ampicillin and amoxicillin, while in New Delhi, 40 Escherichia coli were resistant to gentamicin [15]. In the studies conducted by Jabroldini et al. on 1300 urine samples of outpatients in Amir Al- Momenin Ali (AS) Hospital in Gerash city in the first 6 months of 1396 (2017), it was shown that the culture of 247 samples was positive, of which 108 urine samples were positive in terms of Escherichia coli. The highest and lowest antibiotic resistance were nalidixic acid and nitrofurantoin, respectively [16]. The results of all studies are different from the present study. In general, the prevalence of this type of enzyme is increasing in most geographical areas and one of the most important reasons could be the arbitrary use of beta-lactam antibiotics. The amount of extended-spectrum beta-lactamase (ESBL) varies in strains isolated from different countries and also in one country from one hospital to another, which depends on the infection control system and treatment regimen [17, 18]

In the present study, the size of silver nanoparticles according to the TEM and FESEM images were both 16 nm in diameter.

Salehi *et al.*, in 2016 synthesized silver nanoparticles using Artemisia marschalliana Sprengel plant extract and the results of this study showed that the synthesized nanoparticles have a size of less than 50 nm and have anti-cancer and antimicrobial effects [19]. In 2017, Khalili et al. synthesized silver nanoparticles using Artemisia tournefortiana extract. The results of this study showed that the synthesized nanoparticles have an average size of 22.89 nm and have significant antimicrobial and anti-cancer properties [20].The synthesis of silver nanoparticles using the plant extract of Artemisia annua was reported by Sao Gouda et al. In 2014. In this study, they studied the bacterial and anti-enzyme effects of silver nanoparticles tyrosinase. The researchers stated that silver nanoparticles synthesized using plant extracts as a powerful reducing agent are cost-effective [21].

Lactobacilli are part of the probiotic microorganism and prevent the establishment of many harmful bacteria by producing organic compounds such as lactic acid, hydrogen peroxide and acetic acid and increasing intestinal acidity. Probiotics increase resistance to infection by helping the immune system function. Among the prebiotics and bacteriocins produced, it has a special place among scientists and producers of food such as yogurt [22].

Today, the presence of lactic acid bacteria in fermented milk [23] including yogurt has been shown to have beneficial effects on human health, including balancing the body's natural microflora, resisting the colonization of pathogenic bacteria [24], serum cholesterol levels [25], preventing mutagenicity of agents in the intestine and reduction of intestinal tumors [26], preventing Helicobacter pylori infection [27], urinary tract infections and especially acute gastric and intestinal inflammation [28], reducing lactose intolerance [29] and helping absorb calcium in the intestines. Since yogurt is used orally, the replacement and establishment of lactic acid bacteria in yogurt in the intestine can help maintain the health of the intestine by preventing the growth of pathogenic bacteria by producing antimicrobial compounds and lowering the pH [30].

Among the important properties of nanosilver particles, we can mention very high and fast impact, high stability, environmental compatibility, heat resistance, non-creation and increase of resistance and compatibility of microorganisms [12, 13]. Due to the antibacterial properties of nanosilver and the lack of accurate information about the dose and time of their specific effect on Escherichia coli, detailed tests to follow these effects seem to be necessary. Recent research by scientists shows when nanosilver particles face bacteria and fungi, they disable their respiratory system [10]. This disrupts their cellular metabolism and prevents their growth [23, 24]. In general, studies have shown that nanoparticles themselves has the capability of penetrating bacteria. This capability may be due to the induction of morphological changes in the cell wall, which results in intracellular material settling out and causing bacterial death. Examination with SEM indicates the presence of cavities and holes in the cell wall of Escherichia coli bacteria that have been exposed to nanoparticles [23]. Therefore, it is suggested that the synthesized silver nanoparticles have a small and spherical size that easily cross the cell membrane width and cause cytotoxic effects in bacteria, therefore, with more studies, synthesized silver nanoparticles can be used as a candidate drug and antibiotic for the purpose of microbial infections.

CONCLUSION

The results of the present study showed that silver nanoparticles using yogurt lactobacillus flora alone have good antimicrobial properties and also increase their antimicrobial activity in combination with other antibiotics. And given that Escherichia coli resistant to a variety of antibiotics is increasing, the use of nanoparticles can be effective in combating this pathogen. The results of the study showed that silver nanoparticles synthesized using yogurt Lactobacillus flora with a diameter of 16 nm have antimicrobial activity against Escherichia coli. Also, due to the effectiveness of silver nanoparticles using yogurt lactobacillus flora on Ciprofloxacin-positive Escherichia coli, it can be used as a candidate drug against Escherichia coli in the future.

ACKNOWLEDGMENTS

This research has been approved in Ardabil Azad University with the code 162359366. We would like to thank all those who have contributed to this research.

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