Molecular detection of *Serratia marcescens* isolated from dairy products and study of the inhibition effect of some plant extracts (Garlic and Ginger) compared to antimicrobial agents

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**Abstract:** Milk and dairy products are essential and basic food nutritional factor in the diet of human with high value as a portion of healthy food, and one of the most important causes of food poisoning is contamination of milk and milk products with micro-organisms. Therefore, this study was prepared to detect the contamination of dairy products with *Serratia marcescens* that produce prodiginine pigment in milk and dairy products, Samples of (pasteurized milk, fermented milk drink, and cheeses with high moisture) obtained from various common establishments, such as supermarkets, dairies, and bakeries, from city of Baghdad during 2019 and examined for the presence of *S. marcescens*. From a total of 60 isolates, thirteen (21.6%) isolates that presenting pinkish or reddish color were identified by Microgen™ GnA+B-ID System then confirmed with PCR using 16SrRNA gene with 1500-bp. Seventeen antibiotics belonging to 11 classes, were used to determination of antimicrobial resistance profile, all isolates demonstrated at least one antibiotic resistance from the 17 tested. The most frequently observed resistance was to Cephalothin, was observed in 100% of the isolates. The antibacterial activity of both aqueous plant extracts for garlic and ginger inhibited the growth of the tested isolates. The largest inhibition zone diameter was 42 mm that watched with ginger with concentration of 1000%, compared with garlic that given 38mm. While the lowest inhibition haloes diameter against *S. marcescens* with concentration of 250% was 23mm with garlic and 22mm with garlic and the MIC was 12.5 mg/mL.

**Keywords:** prodiginine pigment, Plants Extracts, Minimum Inhibitory Concentration (MIC), garlic and ginger

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1. **Introduction**

Milk and milk products are widely consumed by humans and consider a proper medium for the rapid growth and multiplication of microorganisms at the proper temperature, Msalya, (2017). It is important to apply considerable care in the production with handling of dairy products to counter any external contamination and to control the growth of organisms through transportation and storage of the product. The appearance of these organisms in these products indicated not only unsanitary conditions but also the yardstick to measure the quality of the products, Bauman *et al.* (2018)

*Serratia marcescens* are capable of producing a pigment at temperatures below 37°C which are called prodigiosin, a component of the prodigin family consisting of linear tripyrrolic cyclic compounds, [Panesar, Kaur, and Panesar (2015)](http://doi.org/10.36295/ASRO.2020.231388). These pigments vary from a dark red colony to pale pink color which depends on the age of the bacterial colonies, *S. marcescens* growth is weak on food that rich with starch and the pigment synthesis began later in the presence of low concentrations of NaCl, but that prodigiosin accumulation/biomass unit was expanded at NaCl concentrations of up to 5%, where the colonies are easily pigmented and resemble the drops of blood, Ochieng *et al.* (2014)

The production of Pigments is unusual manifest during infection; this reason is belong to the normal temperatures of human body that inhibit of production of pigment production. *Serratia* is proficient in growing in various environments, such the digestive tracts of different animals and water in addition soil, Casolari *et al.* (2005)

The Italian pharmacist Bartolomeo Bizio was the first described the unusual ability of the microorganism to produce these pigments, Casolari *et al.* (2005), in 1819 when proved that the “blood” miraculously that develop on stored polenta was absolutely due to the appearance of a living organism which...
is characterized lately as a motile gram-negative bacilli relating to the family of *Enterobacteriaceae* with lactose fermenting, catalase-positive, and produces an extracellular DNase. *S. marcescens* can survive and grow under anaerobic conditions with very low quantity of organic material required, Iguchi *et al.* (2014).

The presence of *S. marcescens* in a food should be considered a cause for concern because this bacterium, which has been once commonly regarded as harmless to the gastrointestinal tract, Societa *et al.* (2018), has been identified in recent studies as a potential pathogen, capable of causing effects of cytotoxicity, inflammation, and invasion, similar to those produced by classic enteric pathogen, Mahlen, (2011).

The treatment of *S. marcescens* infections is very difficult because there are many recently isolated strains of *S. marcescens* with a high level resistance to various antimicrobial agents, lead to the increase of resistance during therapy and these strains are usually carried inducible AmpC β-lactamases chromosomally encoded, especially when treatment with cephalosporins as a broad-spectrum. This ability should be considered when selecting antimicrobial agents for the treatment the infections of *S. marcescens*, Ochieng *et al.* (2014).

Lately found that Serratia spp. commonly found in commercialized dairy products besides the pathogenic microorganism such as *Salmonella enterica* and *Acinetobacter sp* in addition to *Escherichia coli*, which were always the targets of the studies. In this work, our objective was to isolate *S. marcescens* found in dairy products purchased in Iraq, Baghdad, and investigates the inhibition effect of some plant extracts compared to antimicrobial agents.

2. Materials and methods

Sample collection

Samples of commonly consumed dairy products in Baghdad City (pasteurized milk and fermented milk with high moisture cheeses) obtained from various commercial outlets, such as dairies, supermarkets, and bakeries. Each sample was conditioned in coolers containing reusable ice packs, in the original packaging of each product and delivered to analysis at most within two hours after purchase. Samples were diluted into sterile peptone water 0.1% (wt/vol) (Himedia, India). After homogenization, serial dilutions were prepared in test tubes containing the same diluent. Aliquots were inoculated on plates containing selective media (Tryptic Soy Agar and MacConkey agar, Himedia, India). In 37 °C the plates were incubated with 18-24 h.

Bacterium identification and storage

Isolates presenting pinkish or reddish color on Tryptic Soy Agar plates and MacConkey agar were selected and submitted to Gram stain and oxidase test. The isolates of Gram and oxidase-negative were identified by System of MicrogenTM GnA+B-ID that applied for identification of *Enterobacteriaceae*. For maintenance of the isolates, Bacteria were spread over Tryptic Soy plates Agar and incubated for 18-24h at 37 °C. The growth of each isolate was harvested and transferred to 1.5 ml of BHI broth (wt/vol) (Merck, Germany) followed the addition of 40% glycerol (vol/vol) (Merck, Germany) mixed by inversion and stored at -20°C until use, Quinn *et al.* , (2011).

Molecular characterization by 16SrRNA

Bacterial DNA extraction

According to the manufacturing methods, the Wizard genomic (QIAGEN DNA purification kit, Germany) was used as DNA purification kit for extraction the genomic DNA of isolated bacteria.

Specific amplification of 16S rRNA by using PCR

The full length of the 16S rRNA gene (1500-bp) was amplified, as shown by (Sambrook *et al.* 2001) by the polymerase chain reaction (PCR).

The universal primers used for 16S region in Eubacteria amplification are Start as a forward primer (Bac8F): 5’ AGA GTT TGA TCC TGG CTC AG3’ and the End of backward primer (1392 R): 5’ GGT TAC CTT GGT AGC ACT T 3’.

The PCR reaction was done as follow; a total volume of (50 μl) that containing (5 μl) of 10 x buffer, (4 μl) of 25 mM MgCl2, (4 μl) of 2.5 mM dNTPs, (2 μl) of 10 pmol forward primer, (2 μl) of 10 pmol backward
primer with (2 μl) of 50 ng of bacterial genomic DNA and 0.4 μl (5 units/μl) of the Taq DNA polymerase (Promega, Germany). The PCR amplification was performed in a thermal cycler (Eppendorf, Germany) extended for a cycle at 95 °C for 5 minutes, followed by 30 denaturation cycles at 95 °C for 45 s, followed by annealing at 50 °C for 1 min and elongation at 72 °C for 2 minutes. For terminal extension, the reaction mixture was incubated at 72 °C for 10 min [Tariq and Prabakaran (2010)].

16S rRNA-gene sequencing

An Automated Sequence of DNA was used for DNA sequencing with terminator dye (Macrogen Company, Korea) and then analyzed with the gene bank by the nucleotide blast for the nucleotide matching, and the sequenced isolates were pasted and analyzed in the FASTA format, Caporaso et al. (2011).

Antibiotic resistance profile

The antimicrobial resistance profile of isolates was calculated as suggested by the Institute for Clinical and Laboratory Education, using Bowser-Bauer diffusion process, CLSI, (2014) for bacteria of the Enterobacteriaceae family. Seventeen antibiotics (Laborclin, Paraná), belonging to 11 classes, were used: amikacin (30 μg), amoxicillin-clavulanic acid (20/10 μg), ampicillin (10 μg), aztreonam (30 μg), cephalothin (30 μg); cefazidime (30μg) cefotaxime (10 μg), ciprofloxacin (10 μg), chloramphenicol (30 μg), streptomycin (10 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), norfloxacin (10 μg), tetracycline (30 μg), tobramycin (10 μg) and trimethoprim (10 μg).

Plants Extracts Preparation

Plants extracts were carried out in present study using tow common plants, Allium sativum (Garlic) and Zingiber officinale (Ginger) which were collected from local market of Baghdad - Iraq. The first step in this study was rinsing by tap distilled water to remove impurities such as dust. Then, it was grinded for about Five minutes, ten gr of grinded powder was taken and mixed with (100 ml) distilled water then mixed with an electric mixer for half an hour. The extracts were filtered by muslin cloth, then by filter paper (Whatman No 1), Centrifuge at a speed of 3000 rpm / min for 10 minutes, and then filter the extract using filter paper to obtain a clear solution. Dry the extract using an oven at 40 ° C and store in the refrigerator at 6-4 ° C until use.

Antimicrobial activity

Sterile distilled water has been used to dilute extracted plants to prepare the antibacterial activity of the aqueous plant extracts. concentrations of 250% (v/v) to 1000% (v/v) were provided and Mueller-Hinton agar was poured toward sterile Petri-dishes using Agar well diffusion technique, 100μl of the tested antibacterial agent (extracted plants) was taken to fill up the wells holes that doing by sterile cork-borer to get uniform wells in the inoculated agar. The temperature of 37°C was used to incubate the Petri-dishes within 18-24 hrs. Following the incubation period, the zones of inhibition diameters were measured within millimeters (mm) [Clinical and Laboratory Standards Institute (2014)].

Minimum Inhibitory Concentration (MIC) of plant extracts

Serial doubling dilutions with a concentration range of (50, 25 and 12.5) mg / mL were prepared using (Broth Microdilution Method) to prepare the extract plant MIC on a 96-well microtiter plate. All tests were carried out with Mueller Hinton Broth supplemented by Tween 80, briefly, 10 μl of both extracted solution and Mueller Hinton Broth were applied to each well. Then, to reach a concentration of (104 CFU / ml), 10 μl of bacterial suspension (106 CFU / ml) was taken up. To ensure the bacteria did not get dehydrated, the plates were loosely coated with cling film. The plates were 18–24 hours incubator at 37 ° C with prepared in triplicates; the tests were assessed visually by color change. The MIC value was measured at the lowest concentration the change of color occurred at. To generate the MIC values for the analyzed sample, the mean of 3 values was calculated. The MIC is defined as the lowest extract concentration in which the micro-organism does not show noticeable growth, Kleymann et al. (2004)
Phytochemical profile of plants extracts

The active ingredients of garlic and ginger were screening by using the following groups: flavonoids, using Shinoda and the technique of alkalis hydroxide reaction; precipitation process of copper acetate and gelatin with lead acetate, iron salts with tannins, alkaloids; alkaloids during the Mayer precipitation with Dragendorff Bouchardat reactive and Bertrand, and detection the steroids by Liebermann-Burchard response also detection the saponins, within excitement of aqueous extract with determined the foam production within immediate Borntrager reaction with anthraquinones.

Statistical analysis

The results were statistically estimated with 5% of Scott-Knott essay significance and variance analyses (ANOVA), Software Sisvar version 5.3, (2010).

3. Results and discussion

Twenty (33.3 %) were isolated from a total of 60 isolates, exhibited a pinkish to reddish color following the incubation period, indicative of *Serratia marcescens*. These colorful isolates were transferred to new plates of Tryptic Soy Agar and incubated at room temperature, protected from the light for 18h and then, an intense red color was observed in 13 isolates. The 13 (21.6%) isolates were confirmed as *S. marcescens* by Microgen® GnA+B-ID System.

The colonies appeared lactose fermentor On MacConkey agar, with red colonies duo of *Serratia marcescens* ability to produce a pigment, as shown in Fig. (1) Those findings agreement to Jawetz, (2007). Isolated bacteria formed under light microscopic lenses with gram-negative rods.

*Serratia* isolate colonies were recognized on medium platelets of nutrient agar following the incubation at 37°C, the Colony morphology on nutrient agar revealed as pigmented with the smooth circular entire colony. Measuring growth on Nutrient Agar optimized the growth conditions. Maximum growth was reached at 32°C with pH 7 on the Nutrient broth. After 24hr of incubation, the diffusible Red color prodigiosin produced was recognized.

![Fig. 1: Growth of Serratia marcescens on the tested media with prodiginine pigment](image)

Microgen® GnA+B-ID System performed biochemical tests to classify the strain, and the isolate showed negative results for methyl red and indol test, positive for citrate utilization and voges-prosker test. These conclusions concern the biochemical character of *Serratia marcescens* as defined, Microgen® GnA+B-ID System was used in another study, Doaa, (2019) for detection of Enterobacteriaceae.

In addition to studying the biochemical reactions for the organism, multiple sugar fermentation experiments were carried out. This isolate was capable of fermenting fructose, sucrose, and glucose, while it was unable to ferment lactose; these findings are consistent with the literature published on *Serratia marcescens* as shown in Fig. (2 A and B).
Identification of bacterial strain by 16S rRNA Gene

The 1500 bp PCR product DNA sequencing of the 16S rRNA gene was subjected. The PCR product gave an amplicone with right molecular weight (1500bp) (Figure 3).

The ranges of purity of extracted DNA out of 1.7-2. 350 nm U.V was used for visualized the extracted DNA followed by electrophoresis with 1% agarose gel by 70 volts within 30 min.

The genomic DNA of isolates have been detected with 2.0% of agarose gel electrophoresis which dyed via red safe stain and electrophoresed in 70 volts about 1:30 hr, the 15 lanes in figure (3) have been captured with ultraviolet 350 nm (UV) transilluminator with size of band of (1500) bp plus (100) bp as DNA ladder and this result was reported previously by (madani et al., 2011) study and confirmed by Redondo-Bravo et al. (2018)

DNA sequencing for the amplified (1500bp) 16SrRNA gene

The obtained sequence was submitted to BLASTN in order to find homology with other bacterial 16S rRNA sequences. The PCRamplicone of the 16S rRNA was subjected to DNA sequencing and the sequence analysis revealed that, the nucleotides DNA sequence was closely similar to Serratia marcescens.

The differentiation of the 16S rRNA gene permits comparison at the genus level between organisms of bacteria, as well as to classifying isolates at multiple levels.
The 16S rRNA gene sequence was noticed by Aml et al. (2017) who have analyzed of 5.0 isolates of Serratia contain 99% nucleotide sequence comparable to S. marcescens despite its varied considerably in prodigiosin generation.

![Amplification of 16S rRNA gene with 1500 bp with 70 volt on agarose gel of 2% with TBE buffer for 1:30 hours. L (100): DNA ladder, lanes (1-13), with U.V light (350 nm).](image)

The anti-biogram report generated by the ABIS Online Antibiogram software indicated that the isolates were susceptible to only 4 of the antibiotic which observed in 100% of the isolates, whereas it is resistant and intermediate susceptible to the other.

(Table 1 and Fig.4) showed that all isolates expressed resistance to at least one antibiotic of the 17 tested. The most frequently observed resistance was to Cephalothin, resistance to this drug was observed in 100% of the isolates. The second most commonly observed resistance was against Ampicillin, which 84.62% of isolates were resistant to this agent. For Amoxicillin, 61.53% of isolates were resistant and resistance to Cefotaxime, and Chloramphenicol, Limipenem, while susceptible to Tetracycline, Trimethoprim, Aztronam, and Ciprofloxacin.

Nine (69%) S. marcescens isolates expressed multidrug resistance to the antibiotic tested, since they were resistant to one or more antibiotics of at least three different classes these results were according to Magiorakos et al. (2012) and Raghad et al. (2011).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin(30mg)</td>
<td>0</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Tetracyclin(30mg)</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Imipenem(10mg)</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Gentamicin (10mg)</td>
<td>0</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Aztreonam(30 μg)</td>
<td>13</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin dick(10mg)</td>
<td>13</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Amoxycillin-Clavulanic acid disc (20/10mg)</td>
<td>0</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Chloramphenicol (30mg)</td>
<td>0</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Cefotaxime (10mg)</td>
<td>0</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Ampicillin(10mg)</td>
<td>0</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>
According to [Tekiner and Özpinar (2016)], studies involving MDR bacteria in foods are still very scarce and need to be further investigated. In dairy food, specifically, these MDR bacteria can originate from contaminated milk (bovine mastitis cases, for example), pasteurization problems or even from a post-pasteurization contamination.

Antibiotics are often used excessively and indiscriminately to treat bacterial infections, and prevent infections, Murphy et al. (2016) and this routine may be causing great impact on the food industry, Rolain, (2013). The use of antibiotics for growth promotion in animals is still very debatable. Some lines of thought argue that the consumption of products derived from these animals do not pose risks to human health,

**Antimicrobial activity evaluation of plant extracts:**

According to results that displayed in Table 2, the aqueous plant extracts of garlic and ginger inhibited the growth of the tested isolates. The four concentrations that used inhibit the tested bacteria with deferent diameter. The largest inhibition zone diameter was 42 mm that recognized with ginger with a concentration of 1000%, the diameter of inhibition haloes was compared with garlic that given 38mm. While the lowest inhibition haloes diameter against *S. marcescens* with a concentration of 250% was 23mm with ginger and 22mm with garlic. The aqueous extract inhibited the growth of ten (76.9%) of isolates. When compared between the concentrations of growth inhibition, the normal significant, were (p < 0.05).

The values of the aqueous extract of ginger and garlic ranged from (50-12.5 mg / ml), and by comparison with the MIC determination it was found that MIC was 12.5 mg / ml.

With the extracts evaluated by the agar diffusion process, The aqueous extract of ginger displayed the greatest average growth in inhibition haloes relative to the microorganisms used in this analysis; followed by garlic extract that statistically revealed the specific average development of inhibitory-haloes.

**Table 2:** Diameter of plant extract inhibition zone on target Bacteria with MIC

<table>
<thead>
<tr>
<th>Plant</th>
<th>Concentration of plant extract With inhibition zone diameter on target Bacteria (mm)</th>
<th>MIC (mg/ml) (50, 25, 12.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000 %</td>
<td>750 %</td>
</tr>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4: Results of sensitivity test of 17 of antibiotics dick toward 13 isolates of *Serratia marcescens*
The phytochemical tests of the ginger’s aqueous extract showed saponin with tannin in its production, while extracted garlic exhibited saponin, tannin with alkaloid similar to the results of Martins et al. (2000) and Oliveira et al. (2004). Detected flavonoids and tannins sequentially, without identifying the source of the extract. Regarding the property of the constituents present in these extracts, antimicrobial activity can be associated with the presence of detectable compounds, such as alkaloids, flavonoids, saponins, and tannins; the antimicrobial activity was verified by certain compounds.

4. Conclusion

However, a new analysis will be carried out of the experiments with extracts prepared with other solvents. Chemical composition of the extract is understood to be the result of chemical reagents and the processes used to obtain them and that the active ingredients are typically contained at low concentrations and the raw extract may be diluted further. Because there are several different findings from those found in the literature, we conclude that several factors have affected the study, ranging from climatic conditions and geographical location to the publication of the extract used in the experiment, as well as the genetic variation of some microbial strains. Such findings indicate the need to standardize and improve multiple evaluation methodologies, to indicate and ensure the reliability of the results obtained in antimicrobial assays with plant extracts.

References:


