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The Molecular Identification of Pathogenic *E. coli* Isolated from Raw Cow Milk and Assessment Their Anti-susceptibility to Medical Plants at Al-Najaf city/ Iraq

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Abstract

Background: Toxin-producing Shiga *Escherichia coli* has been identified as a new foodborne pathogen that poses a significant health risk to humans. Shiga toxin-producing *Escherichia coli* can be found in raw cow milk and its derivatives. A small number of *Escherichia coli* strains that produce shiga toxin are pathogenic.

Aim of study: The study aimed to see if there were any virulence genes in 50 milk samples that were typical of Entero-haemorrhagic *E. coli* and evaluate the *Myrtus communis* effects on these bacteria.

Materials and Method: Milk samples were used to isolate E. coli bacteria (n=27), biochemically analyzed, and genetically screened for virulence genes using a multiplex (PCR). The hydro-alcoholic extraction of Myrtus communis leaves was tested at four strengths, ranging from 20-50 mg/ml.

Results: The findings of the molecular profile indicated that (stx2) was found in 11 (40.7%), (hlyA) in 13 (48.2) and eae genes in 9 (33.3) of *E. coli* isolate, respectively. Treatment with an extract of this plant at a dosage of 50 mg/ml had the highest effect on *Escherichia coli*, which was significantly different from all other treatments.

Conclusions: The virulence genes shigatoxin-2(stx2), intimin(eae), and entero-hemolysin (hlyA) were found in Strains of E. coli isolated from milk, according to the findings of this study.

***Keywords*:** *E-coli*, Medical Plants, *Myrtus communis*

Introduction

In the *Enterobacteriaceae* family, Gram-negative bacteria include *Escherichia coli*., lactose fermenting, facultatives aerobic, rods-shaped, non-sporulating, flagellated bacterium (Meng *et al*., 2012). *E. coli* is a common commensal bacterium found in the gastrointestinal tract. (Gomes *et al.*, 2016). It is used as a reliable indication of faecal contamination, manure, soil pollution, polluted water, and milking hygiene violations. (Allocati, 2013). The capacity of *E. coli* strains to generate shiga toxins and other bacterial toxins distinguishes them. (Odonkor and Ampofo, 2013). This virulence factor is exclusive to the *E. coli* enteric infections that produces shiga toxin is defined as shiga toxin-producing *Escherichia coli* (STEC) or *E. coli*

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that produces Vero toxin. *Escherichia coli* (VTEC) (Keseler *et al.,* 2017). Consumption of infected fruits and vegetables, undercooked meat, and unpasteurized raw milk can spread the disease. (Gopal and Kumar, 2013) or direct or indirect faecal pollution of water, direct or indirect interaction with animals, or direct or indirect contact with people (Du and Lutkenhaus, 2017) Based on clinical symptoms in humans, Entero-hemorrhage *Escherichia coli* (EHEC) is a sub-group of shag- toxin-producing *E-coli* (Mirsepasi *et al.,* 2019).

STEC pathogenicity is linked to a number of virulence genes, and genes that code for On plasmids, *E. coli* virulence factors can be discovered., large genomic sections named pathogenically islands (10 to 200 kb), or combined bacteriophages. (Tarchouna *et al.,* 2013). This may be transferred via the horizontal genetransfer method Lambdoid phages carry the genes for shiga toxin 1 and 2 (stx1and stx2), It can be integrated in many copies into the bacterial host genome (Kleinheinz *et al.,* 2014). Cytotoxins encoded by phages are also known as phage-encoded cytotoxins. Shiga toxins are A1B5 toxins that induce vascular endothelial damage in individuals with hemorrhagic colitis and the haemolytic uraemic syndrome by stopping protein synthesis in the host cells, which can lead to apoptotic cell death (HUS) (Malberg *et al.,* 2020). Shiga toxin is a toxin that is produced by Shiga the Intiman factor produced by the eae gene is a kind of *E. coli* that can cling to intestinal epithelial cells and cause attaching and effacing lesions (*E. coli* attachment and effacement) These are found in the pathogenicity island LEE (chromosomal locus of enterocyte effacement). (Da Silva *etal.,*2017).

In shiga-toxin producing *E.coli*, the plasmid-encoded enterohaemolysin A (hlyA) virulence gene is identified. It is responsible for the lysis of blood cells following tissue injury, allowing organisms to multiply more easily. (Badouei *et al.,* 2016).

One of the most common ways for Raw milk and dairy products are used to transmit *E. coli* strains to humans. (Dehkordi *etal.,*2014). Milk contamination is mainly induced by the colonization of pathogenic *E.coli* in the guts of raw cows, which is frequently asymptomatic and goes undetected. The danger of contamination of these ready-to-eat dairy products is exacerbated by poor personal and environmental cleanliness throughout the milking process. (Ranjbar *et al*., 2018). Environmental concerns and the expensive cost of certain antibiotics have fueled a trend to substitute less toxic alternatives. Plant-based products have lately gained a particular place among the many materials used to substitute antibiotics. (Ahmed *et al.*, 2014).

Medical plants have been the only source of pain relief for millennia, and despite advances in science and the creation of synthetic medications, medicinal plants are being utilized on a wide basis today. (Moreira *et al.,* 2014). In recent years, much study has been conducted to examine the antibacterial properties of essential oils and extracts., demonstrating their power and capacity to prevent the growing of a wide range of harmful micro-organisms. (Xu *et al.,* 2017).

Mytaseae family's Myrtus communis is a shrub with multiple stems and branches that is evergreen and fragrant. (Alipour *et al.*, 2014). Several studies have shown that the extract of this plant, which includes terpinolene, cinol, linalol, terpinole, linayl acetate, tanins, and

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flavonoids, has anti-parasitic and anti-infective properties. Its antiviral properties have also contributed to the development of antiherpes simplex drugs. (Hennia *et al.,* 2018) Anti-parasitic and antimicrobial drug resistance has become a serious concern in the globe as a result of unregulated antimicrobial medication usage. Resistance to antimicrobial medicines is such a serious problem that the World Health Organization's theme for 2011 was "Resistance to antimicrobial drugs is a worldwide concern." (Dahmoune *et al.,* 2015).

Microbes may transmit antibiotic resistance from one generation to the next, and even from one microbial species to the next, by creating an antibiotic-resistant gene, and the high levels of infection eventually remained constant despite the use of antibiotics. (Simoneit *et al.,* 2015). Until recently, Several research have been carried out on the antibacterial properties of myrtle leaf and stem extracts against pathogenic microorganisms., with promising results, on *staphylococcus aureus*, *Lactobacillus plantarum*, *Bacillus cereus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, Klebsiella, and Shigella. (Jafri *et al.,* 2014).

Despite the fact that its antibacterial efficacy against specific bacteria types, such as *E. coli*, has remained disputed in several studies., (Edlin *etal.,*2013). Because medicinal plants are so important in traditional treatment, and the fact that these medicines have few side effects on humans, as well as changes in the form of pathogenic bacteria resistance, which necessitates periodic monitoring of antibacterial effects substances, the antimicrobial effects of hydroalcoholic extract of Myrtus communis leaves against the above-mentioned pathogenic bacteria (Kukanur *et al.,* 2015).

Material and Method:

- **Collecting samples:**

  Fifty (50) raw milk samples from different cows were randomly collected between (August 2021 –September 2021), from the four Area of Al-Najaf city/ Iraq at various points. From milk selling center, samples were collected in duplicate in sterile plastic containers. They were tagged and taken to the laboratory in an icebox for quick analysis.

  Bacterial Isolation and Identification:

  For the (mTSBn) broth as an *E. coli* enrichment medium, ten milliliter (10 ml) of milk samples were utilized. On a plate of Levine's Eosin Methylene blue agar, a loopful of the enhanced broth was streaked (L-EMBA). At least 5 *E. coli*-like colonies per plate were chosen after an overnight incubation at 37°C. Presumptive *E. coli* colonies were Gram stained and biochemically detected using the VITIC method (greenish metallic sheen look with dark purple cores). *E. coli* was recovered from 27 of the 50 milk samples.

- **Molecular identification**

  - **DNA extraction:**

    The genomic deoxyribonucleic acid (DNA) was extracted following the procedures provided by the manufacturer GeneAid Genomic DNATMKit (GeneAid Research, USA). The extracted samples of DNA were kept at (-20°C) in preparation for molecular

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investigation using polymerase chain reaction (PCR). (Wang *et al*., 2002). On 17 *E. coli* isolates, multiplex PCR was performed.

Master mix preparation:

The primer sets utilized to identify virulence genes in this work were stx2, eae, and hlyA. (Wang et al., 2002), as shown in Table1.

5.0 l DNA extract, 0.6 mM hlyA, 1.5 mM stx2, and 0.75 mM eae, Master Mix (Bioneer): 20mM TrisHCl (pH 8.9), 50mM-KCl, 30mMNH4Cl, 2.5mM MgCl2, 100units/ml Taq DNAPolymerase, 0.3 nmM each dNT Using nuclease-free water, the total amount of reaction mix was increased to 25 l.

- **Polymerases chains reactions (PCR):**
  Amplification was performed in CFX 1100 (BIORAD systems) using a 3 minute initial denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 25 seconds, annealing at 60°C for 50 seconds, extension at 72°C for 35 seconds, and a final extension at 72°C for 6 minutes.
- **Gel Electrophoresis:**
- At 80 V for 45 minutes, A 1.7 percent agarose gel containing 6 l of 10 mg/ml ethidium-bromide was used to electrophorese five microliters of the PCR result. As a molecular size marker, a fifty (100) bp DNA marker (New England Biolab) was employed. The genes were analyzed using a UV trans-illuminator and the results were recorded on a Gel Documentation System (BIORAD).
- **Study Primers:**

Table (1). Various primer sequences were utilized to identify different *E.Coli* isolates.

Genes	sequences		Product size
Shiga Toxin 2 *stx*2	F	TTAACCACACCCACGGCAGT	346
	R	GCTCTGGATGCATCTCTGGT	
Intimin *eae*	F	GCAAATTTAGGTGCGGGTCAGCGTT	494
	R	GGCTCAATTTGCTGAGACCACGGTT	
Enterohaemolysn *hlyA*	F	AGCTGCAAGTGCGGGTCTG	569
	R	TACGGGTTATGCCTGCAAGTTCAC	

- **Preparation of the alcoholic extract of *Myrtus communis* leaves:**

The leaves of Myrtus communis were dried and crushed before being steeped in 96 percent ethanol for 48 hours at room temperature. The mixture was filtered with a filter cloth after 48 hours to remove the leaves husks. The mixture was then placed in a 5-liter Pyrex and baked for 24 hours at 45°C, where the alcohol was evaporated and a dark brown extract with a soft dough-like consistency was formed. A sensitive scale was used to weigh the extract, The concentrated extract was then transferred to a sterile Petri plate and dried at

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40°C.Dehydrated powders were composed and sterile DW was used to make concentrations of 10, 20, 40, and 80 mg. was stored in a securely sealed glass jar and kept frozen until needed.

After performing specific microbiologic tests to establish the existence of germs, *E. coli* bacteria were produced from samples of patients sent to AL-Saader hospital facility. Until the research began, the cultures were cleaned and kept at room temperature. A The day before the experiments, a little quantity of the mother culture was added to the Hinton broth medium.

The penetrative dissemination technique was used to test the susceptibility of nosocomial bacteria to myrtle extract. To equilibrate the moisture within the plates, the bacterial suspension was soaked on the medium using a 20ml sampler and distributed over the medium using sterile cotton swap. After that, it was dried for five min before being incubated for fifteen min. On the surface of the medium, sterile crude discs with a diameter of mm were put, and microliters of myrtle solution were seeped in at a predetermined concentration on the discs.

Result and discussion.

Table 1, illustrates the results of penetrating the study microorganism with different amounts of *Myrtus communis* extract *E. coli*. The effects of *Myrtus communis* extract on *E. coli* were investigated in a study. Bacteria also revealed that only 40mg/ml (7.81 0.01) and 50mg/ml (7.91 0.03) concentrations have an impact on this strain of bacteria; other concentrations had no effect on the bacteria. Only at the highest concentration did *Myrtus communis* extract have a small effect on *E. coli*. Herbs have been used to treat a variety of ailments. The trust that different generations have in traditional medicine treatment demonstrates the positive impact of this approach. On the other hand, increasing pathogenic bacteria resistance and constant changes in the resistance form of these microorganisms have created major challenges in the use of common antibiotics, necessitating the development of new antibacterial compounds.

Antibacterial, antifungal, antiviral, antioxidant, and antimutagenic activities have been found for *Myrtus communis*, an aromatic and medicinal plant. (Mimica *et al.,* 2010). The effects of numerous plants, including *Myrtus communis,* on *Streptococcus pneumoniae*, *Haemophilus influenzae*, and other bacteria were investigated in a research and *Moraxella catarrhalis* isolated from hospital patients were assessed, and In the diffusion penetration test, the myrtle extract created zones of growth inhibition of 30, 50, and 22 mm. As a result, it was suggested that myrtle extract be used to treat sinusitis and bronchitis. (YAZDI *etal.*,2008). The majority of publications on the effects of extracts on *E. coli* suggest that the myrtle extract had no impact on these bacterial strains, notwithstanding a previous study done in Iran that found a An alcoholic extract of the myrtle plant has a positive impact on *E. coli*.

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Table (2): The inhibition zone diameter (mm) of *Myrtus communis* extract on study microorganism (*E.Coli).*

Concentration of the extract (mg/ml)	*E.Coli*
20	--------
30	--------
40	7.81 ± 0.01
50	7.91 ± 0.03

Table (2) showed the Virulence Factors (*stx2*, *eae*, *hlyA*) Produced by *E.Coli* isolates, PCR was conducted over 27 isolates, using the *stx2*, *eae*, *hlyA* primers to amplify the constitutional genes (*stx2*, *eae*, *hlyA*), and bands were confirmed with gel electrophoresis, isolates gave positive result 40.7% for *stx2* gene with amplified size 346bp , (33.3%) for *eae* with amplified size 494bp and 48.2%) for *hlyA* with amplified size 569bp as show in Table (2), Figure (1, 2 and 3) respectively.

Table (3): Virulence Factors (*stx2*, *eae*, *hlyA*) produce by *E.Coli* isolates.

Genes	+ VE No (%)	-VE No (%)	Number of isolate	Chi-Square ($\chi 2$)
stx2	11 (40.7)	16 (59.3)	27	1.54
eae	9 (33.3)	18 (66.7)	27	1.53
hlyA	13 (48.2)	14 (51.8)	27	1.47
($P \leq 0.09$).				

In this investigation, *E. coli* were shown to be positive for shiga-toxin 2 genes (stx2) in raw cow milk. The presence of Shiga toxin-producing *Escherichia coli* in the milk samples implies that they were contaminated (STEC), a pathogenic *E. coli* strain that can produce shiga-toxins STEC strains have one of the most critical pathogenicity features. Traditional milking practices, which increase the possibility of food pathogens being transmitted into milk samples, may be to blame for the significant contamination of milk and milk products by STEC. (Parisi *et al*.,2010). The lack of a storage facility to keep milk products at a consistent temperature would enhance the rate of pathogen survival and multiplication in these milk samples, as well as the unpasteurized milk product itself and the milker's unsanitary condition. The outcome is in line with the reports. (Al-Zogibi *et al.*, 2015).

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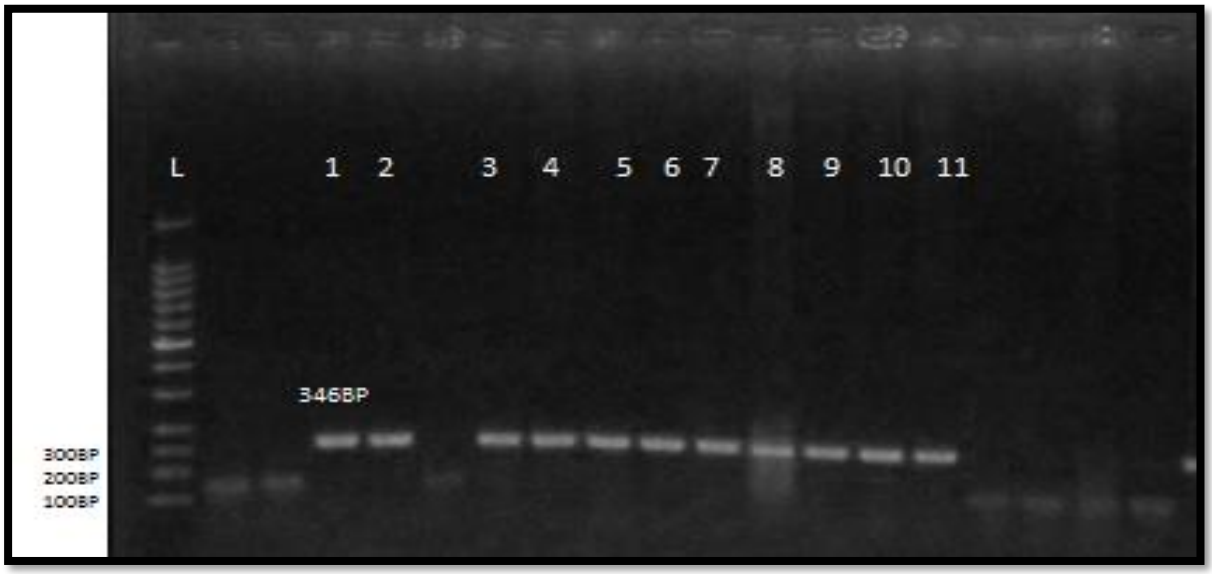

Figure (1): the amplification of *stx*2gene of *E.coli* isolates were fractionated on 1.6% agaroses gel electrophoresis stained with ethidium Bromide. L: 100bp ladder marker. Lanes 1-11 be similar to 346bp PCR products.

The enterohaemolysin hlyA gene was found in 13 (48.2) *E. coli* isolates in this investigation. These findings corroborate the conclusions of the study. (Ntuli *et al*., 2017). This discovery implies that *E. coli* that produces Shiga toxin and expresses the enterohaemolysin gene (hlyA) is a potentially dangerous pathogen in humans, and that its presence in food poses a public health concern. The enterohaemolysin production hlyA gene has been linked to the pathogenicity of a STEC strain's ability to cause more serious illness in people, according to research. (Paton and Paton, 1998; Najim, 2008).

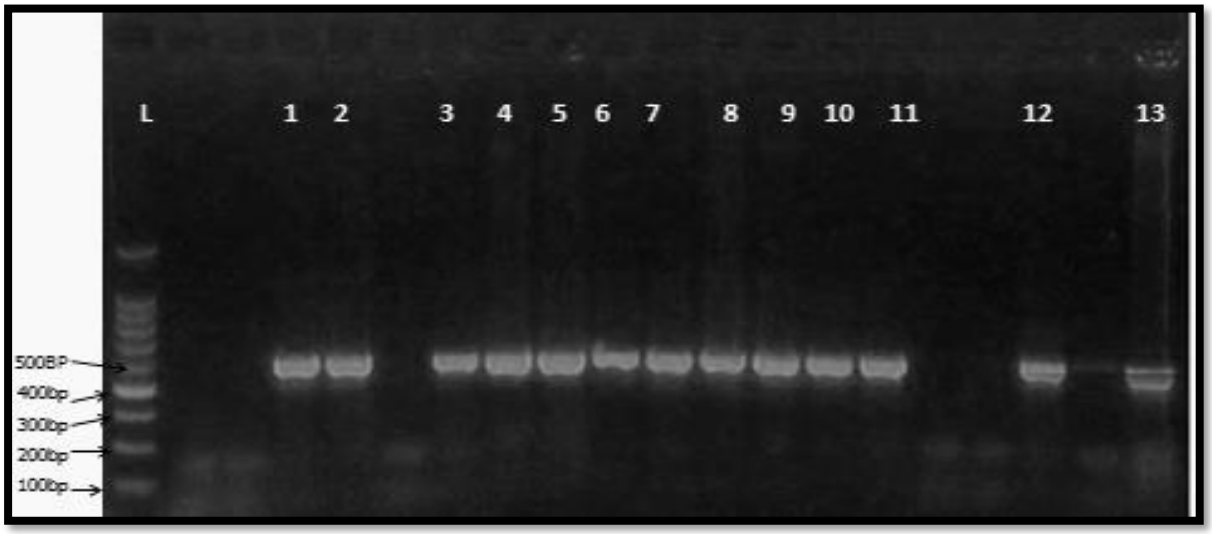

Figure (2): the amplification of *HlyA*gene of *E.coli* isolates were fractionated on 1.6% agaroses gel electrophoresis stained with ethidium Bromide. L: 100bp ladder marker. Lanes 1-13 be similar to 569bp PCR products.

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The intimin eae gene was found in 9 (33.3) of the *E. coli* isolates isolated from milk samples in this investigation. The conclusion is in line with the findings of the investigation. (Momtaz *et al.*, 2012, Dehkordi *et al*., 2014, Ahmed, 2017).

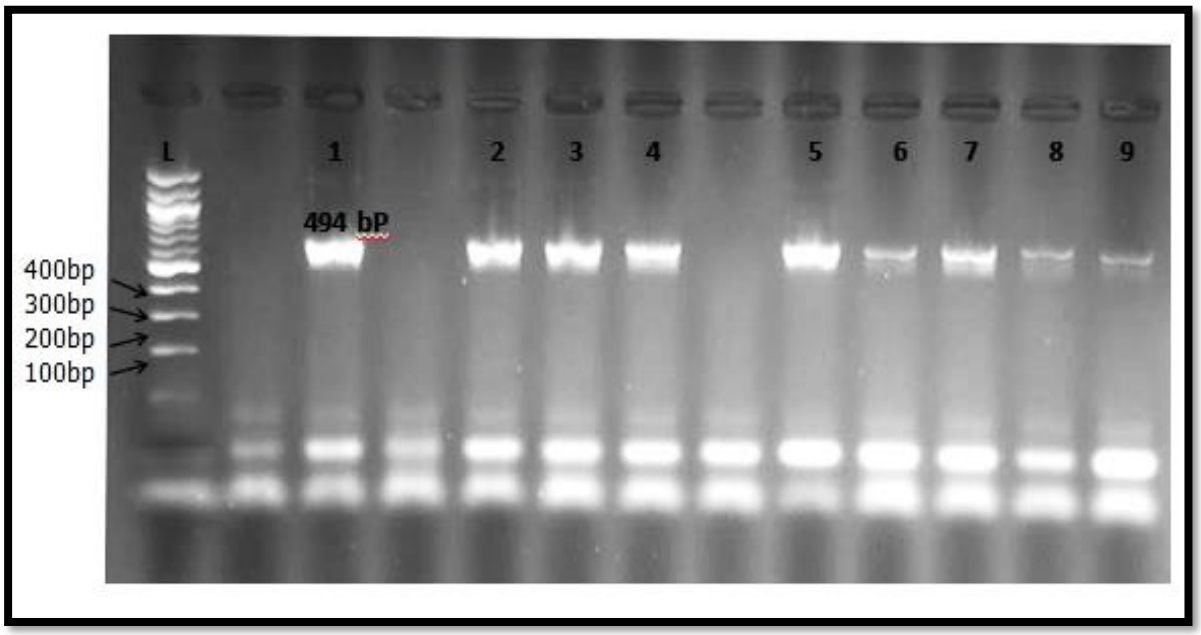

Figure (3): the amplification of *Eae* gene of *E.coli* isolates were fractionated on 1.6% agaroses gel electrophoresis stained with ethidium Bromide. L: 100bp ladder marker. Lanes 1-9 be similar to 494bp PCR products.

Conclusion

- The virulence genes shiga-toxin 2 (stx2), intimin (eae), and enterohaemolysin (hlyA) were found in *E. coli* strains isolated from milk and its products, according to the findings of this study.
- The extracts of *Myrtus communis* used in this study had varying degrees of inhibitory activity against *E. coli.* Bacteria because of the susceptibility of extract-treated cells to the cell wall targeting antibiotic and the appearance of morphological abnormalities, growth suppression was linked to cell wall damage. The extract of the *M. communis* leaf might be a source of chemicals that could be utilized to treat bacterial infections.

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