

Removal of *Listeria monocytogenes* Biofilm with Some Local Plant Extracts of Kurdistan Region, Iraq

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Abstract—The results of removing or reducing biofilm were determined using subinhibitory concentrations (SICs) of some local plants in the flora of Kurdistan region of Iraq on biofilm production in *Listeria monocytogenes*. About 10–500 µg/ml of the plant extracts were used against growth and biofilm production in tissue culture plates which were measured spectroscopically. The SIC effects of each of *Eugenia caryophyllata* (Clove) and *Rhus glabra* (Sumac) have significantly affected on planktonic cells and reduced sessile cells or biofilm formation in *L. monocytogenes*. Remarkably, the data showed strong biofilm reduction under synergistically effect of the plant extract mixed with the antimicrobial agents in multidrug-resistant *L. monocytogenes*. Whereas the SIC some of other plant extracts such as *Salix candida* and pomegranate (*Punica granatum*) have significantly enhanced biofilm expression in *L. monocytogenes*, but the use of these extracts synergistically with some antibiotics also resulted in inhibition of biofilm in comparison to merely use of either the extracts or antibiotics. The result of statistical analyses shows that $P > 0.05$. The results showed that the combination of plant extract with antibiotic together has more effect than using plant merely.

Index Terms—Biofilm, *Eugenia caryophyllata*, *Listeria monocytogenes*, *Salix candida*.

I. INTRODUCTION

Biofilm is an organized aggregate of microorganisms living within an extracellular polymeric matrix that they produce and irreversibly attached to fetish or living surface which will not remove unless rinse quickly (Hurlow, et al., 2015).

Cell-to-cell communication is an important process, during which the required microbial cell density is attained. This leads to the secretion of signaling molecules, known as autoinducers. These autoinducers facilitate quorum sensing. At this stage of maturation, certain gene products are expressed that are considered important for the formation of extracellular polymeric substances (EPS). Since EPS is the main material in the biofilm's three-dimensional structure, interstitial voids are then produced in the matrix (Muhsin, et al., 2018).

Biofilms occur on a wide variety of surfaces including living tissues, industrial equipment, and food processing surfaces such as conveyor belts, plastic, and stainless steel equipment. Since bacterial cells can be easily transferred from biofilms to food products, biofilms formed by pathogens such as *Listeria monocytogenes* are of particular concern for food industries. It has been demonstrated that *L. monocytogenes* can grow and form biofilms on several food processing surfaces including rubber, plastics, glass, and stainless steel. Biofilms of listeria protect cells from the action of antimicrobials and sanitizers, potentially allowing long-term persistence of the microorganism in the food processing environment (Giuseppe, et al., 2013).

Biofilm organisms have an inherent resistance to antibiotics, disinfectants, and germicides. The use of synthetic material for implantation is widely associated with “implant-associated infection” due to biofilm production. In the long run, they may be very damaging due to immune complex disease (Donlan and Costerton, 2002; García-Almendárez, et al., 2007).

Plant-derived compounds have gained widespread interest in the search to identify the alternatives for microbial control (Essawi and Srour, 2000). The compounds are widely accepted due to the perception that they are safe and have a long history of use in folk medicine for the prevention and treatment of diseases and infections (Guarrera, 2005). The present study was focused on using plant extracts at sub-minimum inhibition concentration (MIC) against the clinically relevant *L. monocytogenes* biofilms.

II. MATERIALS AND METHODS

Twelve local plants used in this study. Some of them were obtained from Koya Haebatsultan and others from local market in Sulaimani city.

A. Screening of Biofilm Production

L. monocytogenes isolate that produce biofilm was received from Srwa, 2014, for the current study. Confirmation tests were done through colony morphology and biochemical tests. The isolate was subcultured and the control was prepared in tryptic soy broth (TSB) for 18 h at 37°C. Before the experiments, the strain was vortexed for 5 min and the optical density adjusted to 1–1.5 at 600 nm by spectrophotometer according to McFarland scale to the overnight culture was standardized to a concentration of 3×10^8 CFU/ml.



The strain diluted by obtaining 50 µl of sample to 950 µl broth aliquots of 200 µl transferred to pre-sterilized, 96-well polystyrene microtiter plates commercially available (Deltalab S.L., Spain), then incubated for 24 h at 37°C. After incubation, discard the bacterial suspension totally each well was washed with 200 µl sterile phosphate buffer solution (PBS), then put on the hot plate for 1 h at 60°C for fixation, 25 µl of 1% crystal violet was added to each well, after 15 min at room temperature, each well was washed with 200 µl sterile PBS. The absorbance was determined at wavelength 450–630 nm in an enzyme-linked immunosorbent assay microtiter (Botic England). Controls were performed with crystal violet binding to the wells exposed only to the culture medium without bacteria (Bose, et al., 2009).

B. Plants Classification

The plants were classified by Dr. Eqbal Muhammed Xareb. She is assistant prof. in Koya University/Biology Department and her specialty is horticulture, apple leaves (*Malus domestica*), leaves of *Quercus infectoria*, leaves of bitter vetch (*Lathyrus montanus*), clove (*Eugenia caryophyllata*), and sumac (*Rhus glabra*), *Portulaca oleracea*, *Vitis vinifera* leaves, and *Citrullus colocynthis*, *Anthemis tinctoria* (*Matricaria chamomilla*), *Salix candida*, leaves *Pistacia terebinthus*, and pomegranate (*Punica granatum*).

C. Preparation of Methanol Crude Extracts

The plant samples were collected, and 12 plant extracts were prepared using absolute methanol; add 10 g of grinded plants to the conical flask. Then, 25 ml of absolute methanol was added to the flask then placed in the refrigerator for 3 days. After 72 h, the extracts were filtered through a Whatman filter paper No. 42 (125 mm) and it concentrated using a rotary evaporator (Laborota 4000, SN 090816862, Germany) (Odey, et al., 2012). At the final step, the extracted was transferred into vials and stored at 4°C.

D. Determination of MIC

The minimum inhibitory concentration (MIC) of medicinal plant extracts understudying was determined by turbidity method (spectrophotometric method) at 600 nm, and the following dilutions were prepared for each extract (10, 20, 30, 40, 50, 60, 70, 80, 100, 200, 300, 400, 500, 600, 700, 800, 1000, and 1100 µM) (Ncube, et al., 2008), according to the plant activity, sub-MIC was adjusted. A stock solution of the extracts directly used without dilution, with the control of the bacterial suspension that prepared previously by two methods: 1 – 0.5 ml of the bacterial suspension was put in the wells of the (96-well polystyrene microtiter) plates and the plant extracts was added directly and 2 – 0.5 ml of the bacterial suspension of *L. monocytogenes* was put in the wells of the microtiter plate and was incubated for 24 h to allow set the sessile or biofilm formation. Then, it was mixed with the plant extracts at sub-MIC for each of the 12 extracts. Each well was compared with the control well (Djordjevic, et al., 2002).

E. Susceptibility Test

Antibiotic susceptibility test was conducted for biofilm that produces by *L. monocytogenes* isolate and was used eight extra high-performance liquid chromatography (Sigma) antimicrobials powder that has 100% activity. The bacterial strains were inoculated into TSB and incubated for 24 h at 37°C to allow forming biofilm; then, MIC of antimicrobial agents was added to microwells after discard the bacterial suspension (NCCLS, 2000). The antimicrobial agents that were used [ampicillin (AMP), chloramphenicol (CHL), nalidixic acid (NAL), Rifampin (RIF), Tetracycline (TET), Carbincillin (CAR), Streptomycin (STR), and neomycin (N)].

F. Combination Effect of Antimicrobial Agents with Some Effective Plants on Biofilm

The extracts at sub-MIC were mixed with antimicrobial agents at sub-MIC in sterile Eppendorf tubes. The mixtures were transferred into the wells of the 96 microtiter plates which contain biofilm of *L. monocytogenes* isolate, to determine the nature of the interactions between the extracts and antimicrobial agents using Equation 1 (Srwa and Shwan, 2017).

$$\frac{MIC_{xy} \text{ in combination}}{MIC_x \text{ alone}} + \frac{MIC_{xy} \text{ in combination}}{MIC_y \text{ alone}} \quad 1$$

G. Statistical Analyses

Z score was done for double OD before 10 h incubation and after 10 h incubation. The result was 1.58 from Z score and $P = 0.11$. The result shows that $P > 0.05$ (Richard and Morris, 2000).

III. RESULTS AND DISCUSSION

A. Reducing Biofilm Formation in *L. monocytogenes* by 12 Different Types of Plant Extract

Twelve different types of plant extract were screened for their antimicrobial activities against both planktonic and sessile or biofilm bacteria. First method: The extracts were initially tested on planktonic microorganisms using the MIC assay. The results showed clove (*E. caryophyllata*), *A. tinctoria* (*M. chamomilla*), *S. candida*, leaves *P. terebinthus*, and sumac (*R. glabra*); crude extracts have good effect on inhibiting *L. monocytogenes*. About 0.5 ml of the bacterial suspension from control sample was mixed with the crude methanol plant extracts 10, 40, 80, 500, and 500 µl/ml, respectively, and incubating overnight, then culturing on nutrient agar. The results show no growth isolated for each of them. Meanwhile, some of the extracts can be used such as supportive media or such as nutrient for bacteria. The study of Nyila, et al., 2012 used 13 different sorts of plant extract against *L. monocytogenes* planktonic cells and showed that epigallocatechin was found to be active against *L. monocytogenes* at a concentration of 0.062 mg/ml. While, (Nzeako and Lawati, 2008) used thyme and clove essential oils as antifungal agents and showed growth inhibitions of more than 25 mm (diameter) up to 1:16

dilution. And showed the minimum fungicidal concentration (MFC) of thyme and clove for all the organisms were $1.9 \times 10^3 \mu\text{g/mL}$ for thyme and $2.5 \times 10^3 \mu\text{g/mL}$ for clove. Second method: Treating *L. monocytogenes* that produce biofilm with 12 different types of plant extracts were tested on sessile cells or biofilm formation after incubating for 24 h and set the biofilm. The results are shown in Fig. 1.

Fig. 1 shows the effect of crude methanol extracts of *Q. infectoria* leaves, leaf of bitter vetch (*L. montanus*), bark of clove (*E. caryophyllata*), and seed of sumac (*R. glabra*). In the well (A3, A9, A11, and C8) (0.191, 0.181, 0.284, and 0.178, respectively), they have good effects on reducing biofilm that produces by *L. monocytogenes*, by adding 35, 35, 8, and 8 μL , respectively, of the extracts at sub-MIC in the prepared well previously by adding bacterial suspension and incubating for 24 h till biofilm set by tested isolate and discard the suspension just remain biofilm in the wells. The results were read by ELIZA and well A1 shows control of *L. monocytogenes* OD reading was 1.192.

On the other hand, some of the plant extracts enhance or increase biofilm formation such as *P. oleracea*, *S. candida*, *P. granatum*, *V. vinifera* leaves, and *C. colocynthis* in the A4, B6, B9, C1, and C2, respectively, and OD reading for each of them was 3.22, 2.50, 2.34, 5.60, and 6.32, respectively, as shown in Fig. 1. The present study in accordance with the study of Sandasi, et al., 2010, used 16 different types of plant including clove against three pathogenic microorganisms (*L. monocytogenes*, *Pseudomonas aeruginosa*, and *Candida albicans*), but peppermint was the only extract that showed the best antibiofilm activity against all the tested organisms on a preformed biofilm and also showed that the inhibition of growth of a preformed biofilm was more difficult to achieve resulting in most of the extracts enhancing the growth of the biofilms. Furthermore, the study of Nyila, et al., 2010, showed listerial biofilms, treated with *Syzygium aromaticum* or *Mentha spicata* essential oils, and exhibited the same biomass (absorbance 0.09) as that of the positive control (ciprofloxacin). It was, therefore, assumed that the antilisterial activity of these essential oils could be attributed to the activity of their major chemical

constituents, eugenol, and carvone. However, surprisingly, these compounds alone caused biofilm enhancement rather than inhibition.

B. Susceptibility Test

Susceptibility test was conducted for biofilm that produces by *L. monocytogenes*, eight widely antimicrobials at final concentration were used (AMP, NAL, N, STR, CHL, RIF, TET, and CAR) in well (A1, A2, A3, A4, A5, A6, A7, and A8, respectively), which demonstrated in Fig. 2. OD reading of biofilm that produces by *L. monocytogenes* was 1.195, but when exposed to the antibacterial agents, the relative activity of the biofilm was markedly higher than that in single-species biofilms and the optical density reading of biofilm was 1.439, 1.293, 1.164, 1.144, 1.163, 1.171, 1.104, and 1.092, respectively, as shown in Table I. The study of Burmølle, et al., 2006, was found to interact synergistically in biofilms formed in 96-well microtiter plates: Biofilm biomass was observed to increase by >167% in biofilms formed by the four strains compared to biofilms composed of single strains.

TABLE I
SUSCEPTIBILITY TEST FOR BIOFILM THAT DEVELOPED BY *LISTERIA MONOCYTOGENES* ISOLATE

Isolate	Control	AMP	NAL	N	STR	CHL	RIF	TET	CAR
<i>Listeria monocytogenes</i>	A9	A1	A2	A3	A4	A5	A6	A7	A8
	1.192	1.439	1.293	1.164	1.144	1.163	1.171	1.151	1.092

AMP: Ampicillin, NAL: Nalidixic acid, N: Neomycin, STR: Streptomycin, CHL: Chloramphenicol, RIF: Rifadin, TET: Tetracycline, CAR: Carbenicillin

TABLE II
THE EFFECTS OF COMBINATION ANTIBIOTICS AND SOME PLANT EXTRACTS ON BIOFILM PRODUCED BY *LISTERIA MONOCYTOGENES*

<i>Listeria monocytogenes</i>	RIF	TET	STR	N	AMP
Clove	B1 0.155	B2 0.192	B3 0.108	B5 0.111	B11 0.179
Pomegranate	B6 0.131	B7 0.091	B8 0.106	B9 0.178	B10 0.124
Sumac (<i>Rhus anacardiaceae</i>)	A12 0.253	B12 0.182	C1 0.118	C2 0.177	C3 0.112
<i>Salix candida</i>	C6 0.075	C7 0.055	C8 0.056	C9 0.091	C5 0.065

RIF: Rifadin, TET: Tetracycline, STR: Streptomycin, N: Neomycin, AMP: Ampicillin

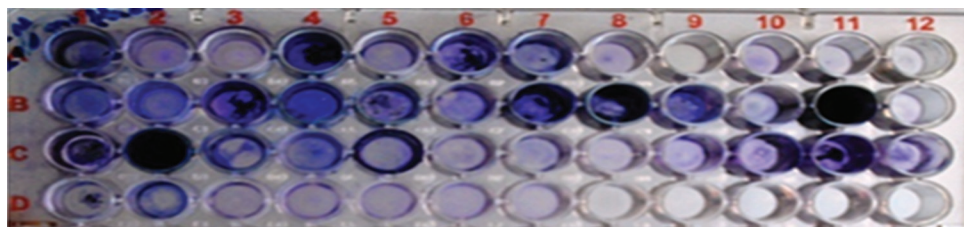


Fig. 1. Treating *Listeria monocytogenes* biofilm with 12 different types of plant extracts.

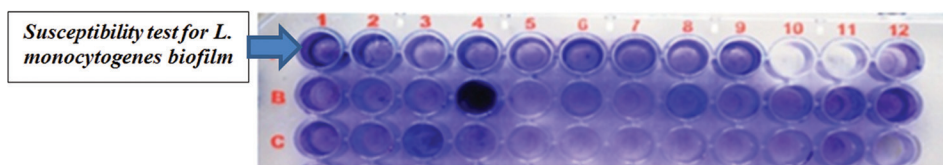


Fig. 2. Treating *Listeria monocytogenes* biofilm with antimicrobial agents and mixed (antimicrobial agents with plant extracts).

When exposed to the antibacterial agent hydrogen peroxide or TET, the relative activity (exposed versus non-exposed biofilms) of the four-species biofilm was markedly higher than that in any of the single-species biofilms.

C. Treating L. monocytogenes Biofilm with Mixed Antimicrobial Agents and Plant Extracts

Combination of five different sorts of antimicrobial agents mixed with three different plant extracts such as clove (*E. caryophyllata*), pomegranate, sumac (*R. glabra*), and *S. candida* separately. They have good effects on reducing biofilm as shown in Fig. 2. The OD reading for each of them demonstrates in Table II. The present study showed synergistic activity between antimicrobial agents and these plant extracts and this agrees with the study of Srwa and Shwan, 2017 showed in their study synergistic activity between plant extracts and antimicrobial agents on biofilm produced by *Klebsiella pneumonia*.

In general, sumac (*Rhus anacardiaceae*) and clove (*E. caryophyllata*) were the only two extracts with low concentration showed good antibiofilm activity and good antibacterial activity against *L. monocytogenes*, while some of the extracts enhanced bacterial activity and biofilm development. And the use of these extracts synergistically with some antibiotics also resulted in inhibition of biofilm in comparison to merely use of either the extracts or antibiotics. There is little study about reducing biofilm by traditional herbs or plants, while many researches nowadays carried out about inhibition of bacterial activity.

IV. CONCLUSION

The results show that the reduction of biofilm mass using plant extracts has good effect, but using the combination of plant and antibiotic together has more effect than using plant solely.

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REFERENCES

Bose, S., Khodke, M., Basak, S., and Malick, S.K., 2009. Detection of biofilm producing staphylococci: need of the hour. *Journal of Clinical and Diagnostic Research*, 3(6), pp.1915-1920.

Burmølle, J.S.W., Dhana R., Lars H.H., Søren J.S.M., and Staffan, K., 2006. Enhanced Biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multi species biofilms. *Journal of American Society for Microbiology Applied and Environmental*

Microbiology, 72(6), pp.3916-3923.

Djordjevic, D., Wiedmann, M., and Mclandsborough, L.A., 2002. Microtitre plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*, 68(6), pp.2950-2958.

Donlan, R.M., and Costerton, W., 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical microbiological Review*, 15(2), pp.167-193.

Essawi, T., and Srour, M., 2000. Screening of some palestinian medicinal plants for antibacterial activity. *Journal Ethnopharmacology*, 70(3), pp.343-349.

García-Almendárez, B.E., Cann, I.K.O., Martin, S.E., Guerrerro-Legrreta, I., and Regalado, C., 2007. Effect of *Lactococcus lactis* UQ2 and its bacteriocin on *Listeria monocytogenes* biofilms. *Food Control*, 19, pp.670-680.

Giuseppe, B., Russo, P., Capozzi, V., Amodio, M.L., Giuseppe, S., and Luciano, B., 2013. *Listeria monocytogenes*, biofilm formation and fresh cut produce. *Food and Environmental Sciences*, pp.114-123.

Guarrera, P.M., 2005. Traditional phytotherapy in central Italy (Marche, Abruzzo, and Latium). *Fitoterapia*, 76(1), pp.1-25.

Hurlow, J., Couch, K., Laforet, K., Bolton, L., Metcalf, D., and Bowler, P., 2015. Clinical biofilms: A challenging frontier in wound care. *Adv Wound Care*, 4, pp.295-301.

Muhsin, J., Wisal, A., Saadia, A., Fazal, J., Muhammad, I., Muhammad, A.N., Tahir, H., Muhammad, A., Muhammad, R., and Muhammad, A.K., 2018. Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association*, 81(1), pp.7-11.

National Committee for Clinical Laboratory Standards (NCCLS). 2000. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*. National Committee for Clinical Laboratory Standards, Wayne, PA.

Ncube, N.S., Afolayan, A.J., and Okoh, A.I., 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: Current methods and future trends. *African Journal of Biotechnology*, 7(12), pp.1797-1806.

Nyila, M.A., Leonard, C.M., Hussein, A.A., and Lall, N., 2012. Activity of South African medicinal plants against *Listeria monocytogenes* biofilms, and isolation of active compounds from *Acacia karroo*. *South African Journal of Botany*, 78, pp.220-227.

Nzeako, B.C., and Lawat, B.A., 2008. Comparative studies of antimycotic potential of thyme and clove oil extracts with antifungal antibiotics on *Candida albicans*. *African Journal of Biotechnology*, 7(1), pp.1612-1619.

Odey, M.O., Iwara, I.A., Udiba, U.U., Johnson, J.T., Inekwe, U.V., Asenye, M.E., and Victor, O., 2012. Preparation of plant extracts from indigenous medicinal plants. *International Journal of Science and Technology*, 1(12), pp.688-692.

Richard, J.L., and Morris, L.M., 2000. *An Introduction to Mathematical Statistics and its Applications*. 3rd ed. Prentice-Hall, New Jersey, p.282.

Sandasi, M., Leonard, C.M., and Viljoen, A.M., 2010. The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria Monocytogenes*. *Letters in Applied Microbiology*, 50(1), pp.30-5.

Srwa, A.M., 2014. Biofilm determination of *Listeria monocytogenes* that isolated from different sources. *Journal of Life Science*, 8(10), pp.1-5.

Srwa, A.M., and Shwan, K.R., 2017. Inhibition of biofilm production in multi-drug resistant *Klebsiella pneumonia* by extract of some plants in the flora of Koya city. *Proceedings ICNS Charmo Journal*, 2, pp.60-67.