IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 10, Issue 2 Ver. III (Mar -Apr. 2015), PP 54-59
www.iosrjournals.org

Reducing Biofilm in Listeriamonocytogenesby Some Plant Extracts

Srwa Ali Muhammed
Department of Medical Microbiology, Faculty of Science and Health, Koya University, Daniel Mitterrand Boulevard, Koya KOY45 AB64, Kurdistan Region - Iraq.
Lecturer with master degree my specialty Microbial genetic

Abstract: *The results of reducing biofilm were determined by using methanol extract for 32different types of plant extracts. Determined the minimum inhibition concentration of these plant extracts, (1 -10) folds it means (100-1000) µg/ml of plant extracts according the plant activity adjusted minimum inhibition concentration (MIC) and then determined Sub minimum inhibition concentration (sub MIC) and used it as reducing biofilm agents by two methods: planktonic cells was treated with these extracts and sessile cells or biofilm was treated by these extracts.Quantification of biofilm reducing was carried out by using a microtiter plate assay and a spectrophotometric method. The result of methanol extracts of {nthemistinctoria (Matricariachamomilla), and Clove (Eugenia caryophyllata,, Salix candida, leaves of Quercusbrantii Oak, leaves of pomegranate (Punicagranatum and Sumac Rhusanacardiaceae)} were showed good antibiofilm activity against (Listeria monocytogenes) in both methods, while the majority of the extracts enhanced biofilm development. Combination extracts with antimicrobial agents showed mainly synergism and interaction was observed on inhibition of the biofilmwhile biofilm that produced by L. monocytogenes was resistance to most of the eight antimicrobial agents ((ampicillin (AMP),naldixic acid(NAL), neomycin (N), streptomycin (STR), chloramphenicol (CHL), rifampin (RIF), tetracyclin (TET) , and carbincillin (CAR) at final concentration used in this study.*

Keywords: *Biofilm,Eugenia caryophyllata, Listeria monocytogenes, nthemistinctoria,Salix candida*

I. Introduction

Biofilms occur on a wide variety of surfaces, including living tissues, industrial equipment and food processing surfaces, such as conveyer 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 monocytogenesare of particular concern for food industries It has been demonstrated that Listeria monocytogenescan 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. This evidence suggests that Listeria monocytogenesbiofilms represent a threat to food safety as bacteria can be transferred to food products when they come into contact with biofilms, For this reasons it appears critical to detect and remove L. monocytogenes biofilms in food processing environments in order to improve food safety[1].In nature, micro-organisms exist as both planktonic, free-floating cells or in a community commonly referred to as a biofilm. A biofilm is a community of cells attached to either a biotic or abiotic surface enclosed in a complex exopolymeric substance (EPS) [2] [3].

Several efficient qualitative and quantitative techniques have been described for rapid and efficient detection of biofilms [4,5] and simultaneous screening of different natural agents for their anti-biofilm potential [6], where, many of these anti-biofilm agents reported are synthetic or of chemical origin[7,8].

.

II. Material And Methods

Thirty two local plants used in this study. They were obtained some of them from KoyaHaebatsultaMountain and others from local market in Sulaimanya city in Kurdistan.Most of these plants daily uses in our society like tea or for boosting meals taste and flavor. In this study, the antimicrobial properties of somecommonly used plants (in the form of extracts) that are reported to be of medicinal value were investigated against the planktonic and biofilm form of Listeria monocytogenes.

II.1 Preparation of methanol crude extracts

The plant samples were collected, and 32 plant extracts were prepared by using absolute methanol; all plants without washing were put in the freezer for one week, then grinded by grinding machine. Add 10g of grinded plants to conical flask. Then 25 ml of absolute methanol was added to the flask then placed in the refrigerator for three days [9]. After 72 hours the extracts were filtered through a Whatmann filter paper No. 42 (125 mm)

and it concentrated by using a rotary evaporator (Laborota 4000, SN 090816862, Germany) with the water bath set at 40°C [10]. At the final step the extracted were transferred into vials and stored at 4°C.

.

II.2 Determination of minimum inhibition concentration

The minimum inhibitory concentration of medicinal plant extracts under studying were determined by turbidity method (spectrophotometricmethod) at 600nm, and the following dilution were prepared for each extract(100, 200, 300, 400, 500, 600,700,800,1000) µg/ml [11], (1 -10) folds of plant extracts according the plant activity adjusted minimum inhibition concentration (MIC) and then determined Sub minimum inhibition concentration (sub MIC). A stock solution of the extracts directly used without dilution with bacterial suspension prepared by obtain a few colonies from the overnight culture of blood agar of L. 'monocytogenes by using (1-42) folds of bacterial suspension according the plants bioactive compounds by two method one time mixed bacterial suspension with the plant extracts. It means with planktonic cell directly. Another method put the bacterial suspension in the incubator for (8-10) hours to allow set the sessile or biofilm formation and then mix with the plant extracts at sub MIC for each of the 33 extracts. Compared each tests well with control prepared by transferring a few colonies from the overnight culture of blood agar (Difco U.S.A.)were transferred into 5ml nutrient broth (Mast diagnostic U.K) medium turbidity of suspension which was adjusted to (1-1.5) through the optical density reading at 600nm by spectrophotometer. The MIC of methanol extracts of medicinal plants is determined through standard curve which prepared previously by reading the optical density of blank and converted to bacterial number[12]. Cell attachment was indirectly assessed using the modified crystal violet assay [13].

II.3 Susceptibility test

The antibiotic susceptibility test conducted for biofilm that produce by L. monocytogenes used eight extra HPLC(sigma) antimicrobials powder that have 100% activity. Tryptic soy broth was used as growth medium, after sterilization and cooling at 45˚C, inoculate the bacterial strains and incubate for 24 hour at 37 ˚C to allow forming biofilm then final concentration of antimicrobial agents were added to micro wells after discard the bacterial suspension. Final concentration of antimicrobial agents were ampicillin (AMP) 50 µg / ml, chloramphenicol (CHL) 10 µg / ml, nalidixic acid (NAL) 30 µg / ml, Rifampin (RIF) 20 µg / ml, Tetracycline (TET) 15 µg / ml, carbincillin (CAR)50 µg / ml, Streptomycin (STR) 10 µg/ml, and neomycin (N) 100 µg/ml)[14].

Determined the effect of mixed antimicrobials with four of the plant extracts at final concentration of both of them. Prepared bacterial suspension and put in the wells for 24 hr. incubation at 37ºC then discard the suspension and add mixed antimicrobial agents and plant extracts separately in each well.

II.3 Statistical Analyses

Zscore was done for double OD before 10hrs incubation and after 10hrs incubation. The result was 1.58 from z score and P value was 0.11.the result shows that P value > from 0.05[15]

III. Result And Discussion

Some research reports show that most spices have antimicrobial properties some suggest that spices provide supportive media for the growth of some bacteria and fungi [16]. In the present study also showed that some of these extracts have antimicrobial properties while the others we can use it such as supportive media for bacteria.

III.1 Reducing or elimination biofilm in L. monocytogenes by 32 different types of plant extracts

AStrain of **Listeria monocytogenes** that produce biofilm and the optical density reading was 0.195 obtained from[17]. Producing biofilm by most of the bacterial strains is a big problem because antibiotic sometimes cannot remove the biofilm although cause to kill the bacterial strain. Biofilm remain on the surface of this infected place as a biomass, especially with those patients that have plastic heart valve or kidney plantation or those who put cannula to take drugs [18]. In this study to reduce these problems thirty threedifferent types of plant were used as shown in Table 1 by two methods: Plant extracts were screened for their antimicrobial activities against bothplanktonic and sessile bacteria.

First method: Table (1) shows the effect of 32 different types of plant extracts used for curing or reducing biofilm in tested isolate. The MIC was determined for plant extract, which inhibited bacterial growth. The test is compared to the control sample that consists of 5 ml of nutrient broth and 0.1 ml of overnight culture of bacterial suspension, and then incubated at 37˚C for 24 hours by using 1 fold to 40fold from the bacterial control suspension mix with 1fold to 10 fold (100-1000) µg/ml to each methanol plants extract according the ability of the plants to inhabit the bacterial activity or to reach the point of minimum inhibition concentration , then

determined sub MIC and used as curing agents by streaking method for L.monocytogenesisolate on nutrient agar.The extracts were initially tested on planktonic micro-organisms by using the minimum inhibitory concentration (MIC) assay.

The results show in "fig. 1"L. monocytogenes that obtained from [17] has high ability to produce biofilm the optical density reading by ELIZA (0.19) as shown in table (1). "Fig. 1"(E11, F8, G4, H6, and H7) shows {nthemistinctoria (Matricariachamomilla) ,, Clove (Eugenia caryophyllata),, Salix candida, leaves Pistaciaterebinthus,,,, Sumac Rhusanacardiaceae)}crud extract were effected on biofilm produced by L. monocytogenes by mixing one fold of the plants with(11, 42, 6, 1 and 1) folds respectively of the bacterial control suspension put it in the incubator under 37°C for 24 hours then biofilm test was done and reading OD of biofilm were (0.080, 0.107, 0.114, 0.100, and0.102) respectively, and the effect of(leaves Quercusbrantii Oak F9, leaves of pomegranate (Punicagranatum) F12, Seed glycyrrhizaglabra G1, and leaves glycyrrhizaglabra G3)were observed by mixing (3,2,4 and 4) folds of methanol plant extracts with one fold of bacterial control suspension and reading optical density were (0.066, 0.068, 0.066, and 0.122) respectively. It means these plants act as curing agentfor prevention of cell attachment and biofilm development by using a little amount of the extracts as demonstrate in table (1).While some of the plants caused to increase or enhance biofilm or bacterial activity as demonstrate in"fig. 1"such as [Eggplant leave (Solanummelongena L.)F4, root of wolfabane Aconitum F7, tolaka H8} were observed by mixing (5, 10, and 6) folds of methanol plant extracts with one fold of bacterial control suspension and reading optical density were (0.413, 0.506 and 0.806) respectively as shown in table (1).

Table (1) the effect of 33 different types of plant and herb extracts on L. monocytogenes

No. of wells	Concentration of Listeria monocytogenes No.7	OD of biofilm after treating with plants	Plants scientific name	Sub MIC of plants (fold)
E7	**Listeria suspension**	**0.386**	**-**	**-**
E8	**1**	**0.210**	**Apple leaves (MalusDomestica)**	**3**
E9	**1**	**0.203**	**pine needles (Pinuspalustris),,,**	**3**
E10	**1**	**0.368**	**PortulacaAleracea**	**4**
E11	**11**	**0.080**	**nthemistinctoria (Matricariachamomilla)**	**1**
E12	**1**	**0.397**	Prosopisfarcta	**4**
F2	**1**	**0.238**	**Okra –leaves (Abelmoschusesculentus L)**	**4**
F3	**1**	**0.302**	**(Qurcusinfectoria olive leaves**	**4**
F4	**1**	**0.413**	**Eggplant leave (Solanummelongena L.)**	**5**
F5	**1**	**0.222**	**leaf of wolfabane Aconitum**	**6**
F6	**1**	**0.190**	**Leaf of Bitter Vetch …LathyrusmontanusLeguminosae**	**4**
F7	**1**	**0.506**	**root of wolfabane Aconitum**	**10**
F8	**42**	**0.107**	**Clove (Eugenia caryophyllata)**	**1**
F9	**1**	**0.066**	**Quercusbrantii) Oak**	**3**
F10	**1**	**0.362**	**Bushy broom grass (Andropogonglomeratus)**	**4**
F11	**1**	**0.147**	**Okra –truck (Abelmoschusesculentus L))**	**4**
F12	**1**	**0.068**	**pomegranate (Punicagranatum)**	**2**
G1	**1**	**0.066**	**Seed glycyrrhizaglabra**	**4**
G2	**1**	**0.196**	**Prunuspersica Peach**	**5**
G3	**1**	**0.122**	**Leaves glycyrrhizaglabra**	**4**
G4	**6**	**0.114**	**Salix candida**	**1**
G5	**1**	**o.475**	**Rose of Bitter Vetch …LathyrusmontanusLeguminosae**	**7**
G6	**1**	**0.358**	**Seed of (Qurcusinfectoria olive)**	**1**
G7	**1**	**0.195**	**Cinnamomumzeylanicum**	**7**
G9	**1**	**0.259**	**Vitisvinifera leaves**	**5**
G10	**1**	**0.229**	**Citrullus colocynths)**	**6**
G11	**1**	**0.136**	**Ficuscarica**	**4**
G12	**1**	**0.173**	Sewak	**6**
H2	**1**	**0.492**	**Linumusitatissimum**	**5**
H3	**1**	**0.644**	Althaeaofficinalis	**4**
H6	**1**	**0.100**	**Pistaciaterebinthus**	**1**
H7	**1**	**0.102**	**Sumac Rhusanacardiaceae**	**1**
H8	**1**	**0.804**	**Tolaka**	**6**
H10	**1**	**0.281**	**Control**	**3 Methanol**

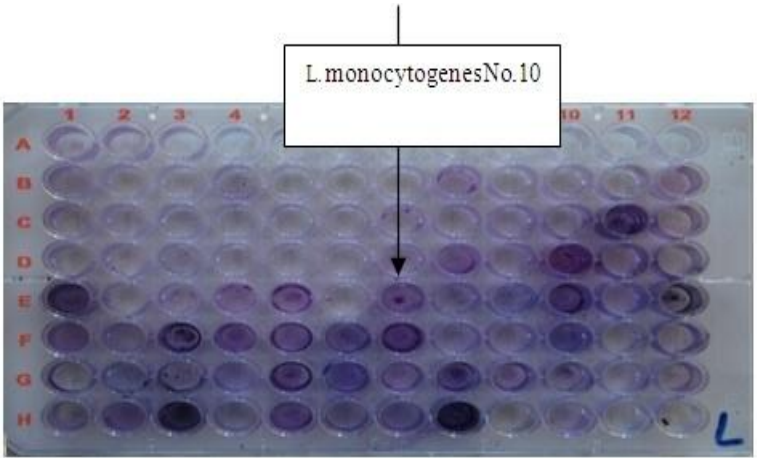
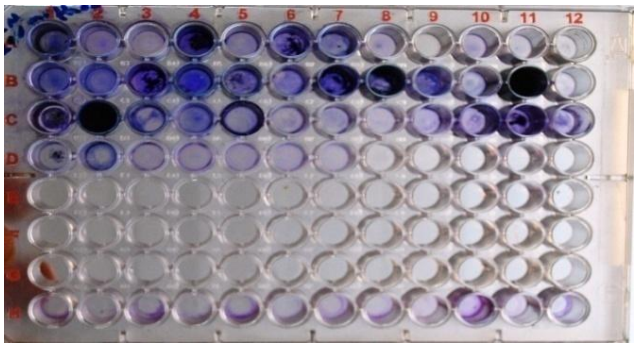


Figure (1) Treating Biofilm that was formed by L.monocytogeneswith 32 plant extracts

Secound method: TreatingL. monocytogenesthat produce biofilm with 32 different types of plant extract were tested on sessile cells or biofilm formation after 8 hour by using the minimum inhibitory concentration (MIC) as demonsitrate in "fig. 2".



Figure(2)TtreatingL. monocytogenesthat produce biofilmwith 32 types of plant extract after set eight hour in incubator

Table (2) the effect of 33 different types of plant extracts on biofilm that produce by L. monocytogenesafter incubation for 8hour

No. of wells	Concentration of Listeria monocytogenes No.7	OD of biofilm after treating with plants	Plants scientific name	Sub MIC of plants (fold)
D1	Listeria suspension	0.195	-	-
A1	1	0.691	Apple leaves (MalusDomestica)	3
A2	1	0.255	pine needles (Pinuspalustris),,,	3
A3	11	0.102	nthemistinctoria (Matricariachamomilla)	1
A4	1	0.821	PortulacaAleracea	4
A5	1	0.353	Prosopisfarcta	4
A7	1	0.194	Okra –leaves (Abelmoschusesculentus L)	4
A8	1	0.121	(Qurcusinfectoria olive leaves	4
A9	1	0.081	Eggplant leave (Solanummelongena L.)	5
A10	1	0.167	leaf of wolfabane Aconitum	6
A11	1	0.084	Leaf of Bitter Vetch …LathyrusmontanusLeguminosae	4
A12	1	0.208	root of wolfabane Aconitum	10
B1	42	0.122	Clove (Eugenia caryophyllata)	1
B2	1	0.101	Quercusbrantii) Oak	3
B3	1	1.172	Bushy broom grass (Andropogonglomeratus)	4
B4	1	0.515	Okra –truck (Abelmoschusesculentus L))	4
B5	1	0.151	pomegranate (Punicagranatum)	2

B6	**1**	**0.141**	**Seed glycyrrhizaglabra**	**4**
B7	**1**	**0.560**	**Prunuspersica Peach**	**5**
B8	**1**	**0.416**	**Leaves glycyrrhizaglabra**	**4**
B9	**6**	**0.632**	**Salix candida**	**1**
B10	**1**	**0.335**	**Rose of Bitter Vetch …LathyrusmontanusLeguminosae**	**7**
B12	**1**	**0.180**	**Cinnamomumzeylanicum**	**7**
C1	**1**	**1.269**	**Vitisvinifera leaves**	**5**
C2	**1**	**1.902**	**Citrullus colocynths)**	**6**
C3	**1**	**0.179**	**Ficuscarica**	**4**
C4	**1**	**0.304**	**Sewak**	**6**
C5	**1**	**0.082**	**Linumusitatissimum**	**5**
C6	**1**	**0.483**	Althaeaofficinalis	**4**
C7	**1**	**0.388**	**Pistaciaterebinthus**	**1**
C8	**1**	**0.078**	**Sumac Rhusanacardiaceae**	**1**
C9	**1**	**0.181**	**Tolaka**	**6**
C10	**1**	**0.178**	**Control**	**3 Methanol**

"Fig. 2"shows {nthemistinctoria (Matricariachamomilla), Qurcusinfectoria olive leaves, Eggplant leave (Solanummelongena L.), Leaf of Bitter Vetch …LathyrusmontanusLeguminosae, Clove (Eugenia caryophyllata), Quercusbrantii) Oak, pomegranate (Punicagranatum), Seed glycyrrhizaglabra, Linumusitatissimum and Sumac Rhusanacardiaceae.)} crud methanol extracts have good effected on biofilm produced by L. monocytogenes but {Clove (Eugenia caryophyllata) and nthemistinctoria (Matricariachamomilla} have more effectiveness on biofilm because one fold of the extract used with(42, 11) foldsof bacterial suspension after incubating for 8 hr.at 37°C respectively then added the extracts on the bacterial suspension and incubation for 24 hr. at 37°C. And the other extracts don't have biofilm reducer activity as shown in table (2).

Table (3) OD reading of bacterial biofilm after treating with antibiotics

Isolate	Control		AMP		NAL		N		STR		CHL		RIF		TET		CAR	
L. monocytogenes	A9	0.195	A1	0.239	A2	0.233	A3	0.164	A4	0.144	A5	0.163	A6	0.171	A7	0.154	A8	0.092

III.2 Susceptibility test

Susceptibility test was conducted for biofilm that produce by L. monocytogenes, eight widely antimicrobials used (AMP, NAL, N, STR, CHL, RIF, TET, and CAR) which demonstrated in table (3) and used at final concentrations by MIC. OD reading of biofilm that produce by L. monocytogeneswas 0.195 but after treating with antimicrobial agents the optical density reading of biofilm were (0.239, 0.233, 0.164, 0.144, 0.163, 0.171, 0.154, and 0.092) respectively and "fig. 3"shows that biofilm resistance to most of the antimicrobial agents and some of them reduced biofilm in a few amount.

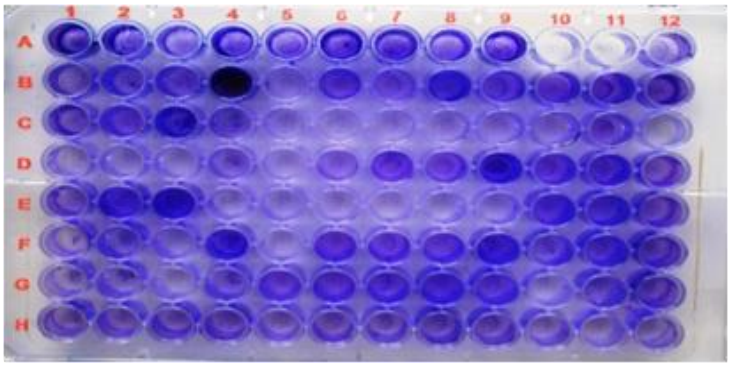


Figure (3)L. monocytogenes biofilm treated with antimicrobial agents and with mixed plants and antimicrobial agents

III.3 Inhibition of biofilm growth using antimicrobial agents with some effective plants in combinatio

"fig. 3''shows the result of four types of effectiveplant extracts mixed with five antimicrobial agents separately and shows most of them have good effect on reducing biofilm as demonstratedintable (4).

Table (4) the effect of mixed antibiotics and some effective plants on biofilm produced by L. monocytogenes

L. monocytogenes	RIF		TET		STR		N		AMP	
Clove	B1	0.055	B2	0.092	B3	0.068	B4	0.611	B11	0.079
Poamgranet	B6	0.131	B7	0.091	B8	0.106	B9	0.078	B10	0.124
Sumac Rhusanacardiaceae	A12	0.053	B12	0.152	C1	0.118	C2	0.177	C3	0.112
Salix candida	C6	0.075	C7	0.055	C8	0.056	C9	0.091	C5	0.065

In generalnthemistinctoria (Matricariachamomilla) and Clove (Eugenia caryophyllata) was the only two extracts that showed good antibiofilm activity and good antibacterial activity against L. monocytogenes while the majority of the extracts enhanced bacterial activity and biofilm development. Also Determined Listeria biofilm resistance to most of the antimicrobial agents but Combination extracts with antimicrobial agents showed mainly synergism activity on biofilm. There are few study about reducing biofilm by traditional herbs or plants while many researches now a day carried out about inhabition bacterial activity.

IV. Conclusion

The resultsshow that the reduction of biofilm mass by use of plant extracts has good effective but using in combination plant and antibiotic together has more effect than using plant alone.

References

[1]. Giuseppe B.*; P. Russo*; V. Capozzi, M. L. Amodio; Giuseppe S.** and Luciano Beneduce (2013). Listeria monocytogenes, biofilm formation and fresh cut produce.University of Foggia, Department of Agriculture, Food and Environmental Sciences, via Napoli, 25, 71122 Foggia, Italy. Phone: +39 (0)881 589303, Fax: +39(0)881 740211, email giuseppe.spano@unifg.it.

[2]. MAH, T.C. & O'TOOLE, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology, 9:34–39.

[3]. HUGO, W.B. & RUSSELL, A.D. (2004). Pharmaceutical microbiology. 7th edition.Blackwell publishing company, USA.

[4]. Merritt JH, Kadouri DE and O'Toole GA. (2005). Growing and analyzing static biofilms. CurrProtocMicrobiol; Chapter 1: Unit 1B .

[5]. Hannig C, Follo M, Hellwig E and Al-Ahmad A.(2009). Visualization of adherent micro-organisms using different techniques. J Med Microbiol; 59(Pt 1):1-7.

[6]. Pitts B, Hamilton MA, Zelver N and Stewart PS. A microtiter-plate screening method for biofilm disinfection and removal. Journal of microbiological methods, 2003; 54(2): 269- 276.

[7]. Cserhati T, Forgacs E and Oros G.(2002). Biological activity and environmental impact of anionic surfactants. Environment international, ,;28(5):337-348.

[8]. Chen X and Stewart PS.(2000). Biofilm removal caused by chemical treatments. Water Research, 34(17):4229–423

[9]. Aiyelaagbe, O.O. and P.M. Osamudiamen,(2009). Phytochemical screening for active compound in Mangiferaindica leaves from Ibadan, Oyo State. Plant Sci. Res., 2: 11-13

[10]. Edeoga, H.O., D.E. Okwu and B.O. Mbaebie, (2005). Phytochemical constituents of some Nigerian medicinal plants. Afr. J. Biotechnol., 4: 685-688

[11]. Cruickshank, R.; J. D. Duguid; B. P. Marmion and R.H.A. Swain (1975). Medical microbiology the practice of medical microbiology. 12thed vol.1 Churchill Livingstone, London and New York.

[12]. heesbrough M (1984) Medical laboratory Manual for Tropical Countries. II. Butterworth-Heinemann Limited;. pp. 33–47. 16–391

[13]. DJORDJEVIC, D., WIEDMANN, M., & MCLANDSBOROUGH, L.A. 2002. Microtitre plate assay for assessment of Listeria monocytogenes biofilm formation. Applied and Environmental Microbiology, 68:2950–2958.

[14]. National Committee for Clinical LaboratoryStandards.(2000).Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee forClinicalLaboratory Standards: Wayne, PA.

[15]. Richard J. Larsen and Morris L. Marx (2000) An Introduction to Mathematical Statistics and Its Applications, Third Edition, ISBN 0-13-922303-7. p. 282

[16]. HOLMES, S.C.H. (2004). The pharmacy on your spice rack: culinary herbs and their medicinal uses. Available on: http//:www.naturalproductsproject.htm.

[17]. Srwa A. (2014). Biofilm Determination of Listeria Monocytogenes that Isolated from Different sources. Journal of Life Science, ISSN 1934-7391,USA doi:10.7265/1934-7391 Vol. 8 No.10, PP.

[18]. MaxleeneSandasi. (2008). The effect of plant extracts on microbial biofilm formation and development. Magister technologiae: Tshwane University Of Technology Supervisor: Prof A M Viljoen Co-Supervisor: Ms C M Leonard.