SUPPLEMENTARY MATERIAL

Purification and identification of 4-allylbenzene-1,2-diol: an antilisterial and biofilm preventing compound from the leaves of *Piper betle* L. var Pachaikodi

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Abstract: Antibiotic resistant food-borne Listeriosis has been rising with up to 30% mortality threat in humans since several decades. Hence, discovering antilisterial from the extracts of ethnomedicinal plants may be of value as a novel antidote. In our preceding study, we reported that ethanolic extract of *Piper betle* L. var Pachaikodi leaves exhibited antibacterial activity towards *Listeria monocytogenes* MTCC 657. Consequently in the present study, the bioactive molecule responsible for anti-*Listeria* activity was purified and identified as 4-allylbenzene-1,2-diol. This identified bioactive compound may have significance while used as antimicrobials and/or food additives in food processing sector as evidenced by dual action: biofilm inhibition and pore formation on cell membrane.

Keywords:
Piper betle L.; 4-allylbenzene-1,2-diol; Antilisterial activity; IEC-6; food supplements/additives; Antibiofilm activity

Experimental

Preparation of crude ethanolic extract

Piper betle L. leaves of Pachaikodi variety purchased from Puducherry local market were authenticated (Kavitha and Jeevaratnam 2016) and the voucher specimen (PU BMB PBvP) has been deposited in the department. The bioactive constituent present in the fresh leaves (100 g) was extracted by 70% ethanol (1:10, w/v) as described previously (Kavitha and Jeevaratnam 2016), yielded 6.505 g of dried residue. Then the residue was reconstituted using 70% ethanol (100 mg/ml) and the crude was stored at 4° C for investigation of antilisterial activity.

Preliminary Phytochemical screening

The predominant promising constituents from 70% TEC extract of betel leaves which showed anti-*Listeria* potency were screened by qualitative chemical tests such as ferric chloride and alkaline reagent tests for the presence of bioactive secondary metabolites such as phenols and flavanoids respectively. These tests were performed on the preliminary basis as per standard methods.

Separation and Purification of bioactive compound

Fractionation of bioactive 70% TCE extracts using Column Chromatography

One gram of bioactive constituent enriched residue (70% TCE extract) was mixed with silica gel (230-400 mesh) from Merck, India. The column (3cm × 18 cm) was packed with silica using hexane: chloroform (100:0) and the sample mixture was loaded on top of the silica. The column was then eluted with solvents of increasing polarity, the order beginning from hexane < chloroform < ethyl acetate < methanol. Fractions of each 100 ml from three different solvent gradient eluent were collected; concentrated using Rotavapor (BUCHI, Flawil, Switzerland) and traces of solvent were removed by Speed Vac (Christ, Germany). Subsequently all the fractions were spotted on precoated TLC silica gel 60 F_{254} plates (Merck) and the spots were analyzed under UV at 254 and 366 nm by CAMAG TLC visualizer (Switzerland). Simultaneously anti-*Listeria* potency was checked for all the fractions at the final concentration of 200 µg/well (10 mg/ml stock) as described in previous study (Kavitha and Jeevaratnam 2016). Ampicillin (2 µg/well) was used as positive control and 70% ethanol as negative control.

Sub and Sub-micro-Fractionation of active fractions

The active fraction (180 mg) that inhibited the growth of *Listeria* was again subjected to silica column (1.5 cm × 20 cm) to ensure complete separation of active constituent from interfering compounds using solvent system- hexane: chloroform with gradient elution ratio of 65:35 to 46:54. The obtained sub-fractions (60 mg) were pooled together based on retention factor and similar pattern on TLC plate. Further purification of the homogenous active constituent (20 mg) from the sub-fractions was achieved using silica column (1 cm × 15 cm) with isocratic system- toluene: ethyl acetate mixture in the ratio of 70:30.

Purification of bioactive constituent using Reversed- Phase Chromatography

The purity of isolated compound was confirmed by HPLC analysis on a Shimadzu instrument (Japan) coupled with a photodiode array detector, using Grace Vydac ^{TM} 218TP reversed phase column (C-18, 5 µm, 250 mm × 4.6 mm). The mobile phase consisted of mixture of solvents: Milli-Q-water (A) and Methanol (B) with gradient starting at 10% B and ending at 100% B after 30 minutes was employed. The isolated bioactive constituent (2 mg/ml) dissolved in methanol was filtered using 0.22 µm filter and twenty microlitres of the sample was injected into the system and the resulting peaks were detected at 254 and 280 nm. Then the single peak representing active compound was purified using semi-preparative HPLC.

Structural elucidation of purified constituent

FT-NMR spectroscopy is an analytical technique used to characterize a compound's molecular structure. Dried eluted compound from semi-preparative HPLC was dissolved in 0.5 ml of $CDCl_3$ and spectral analysis such as 1D- ^{1}H, ^{13}C and DEPT-135; 2D- HSQC, HMBC, COSY and NOESY were recorded on NMR spectrometer model Avance-II (Bruker, Switzerland) with 400MHz frequency using 5 mm multinuclear probes. The accurate molecular mass analysis was carried out using a 6530 Accurate- Mass Q- Time-of-Flight (Q-TOF) LC⁄MS system (Agilent Technologies, Santa Clara, CA, USA) by injecting sample directly. Mobile phase was a 50:50 mixture of solvent A (0.1% formic acid in water) and solvent B (Acetonitrile) at a flow rate of 0.2 ml/min. Mass spectrometer was run in positive electron spray ionization (ESI) mode with mass⁄ charge (m⁄z) ratio in the range of 100–600 m⁄z. Agilent Mass Hunter Workstation Software and Qualitative Analysis B.06.00 were used for data acquisition and data processing. The functional groups are determined using FT-IR spectrum which was recorded on a Shimadzu IR-470 spectrophotometer by employing KBr pellet technique.

In vitro evaluation for the antibacterial activity of 4-allylbenzene-1,2-diol

Bacterial strains

The food-borne pathogenic bacterial strains were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. *Escherichia coli* MTCC 728, *Staphylococcus aureus* MTCC 737, *Listeria monocytogenes* MTCC 657, *Aeromonas hydrophila* MTCC 1739 and *Vibrio parahaemolyticus* MTCC 451 were maintained in Tryptone Soyabean broth (TSB) medium.

Broad spectrum of antibacterial activity

The wide range of inhibitory effect of the bioactive compound 4-allylbenzene-1,2-diol against some food-borne pathogens was determined by broth microdilution method using 96-wells microtitre plates. Both gram positive and negative bacterial cultures used for the assay were sub-cultured in TSB medium and incubated under shaking condition at 37°C for 6 h.

Broth microdilution assay: The MIC was determined as per Clinical and Laboratory Standards Institute guidelines. Bacterial inoculums were prepared for all bacterial cultures in the same medium by adjusted density to 0.01 at 600 nm. Stock concentration (2 mg/ml) of bioactive compound was made by 70% ethanol. The two-fold serial dilutions of 4-allylbenzene-1,2-diol was prepared in 96-wells plates using TSB, 100 µl of inoculums were added to each well of a plate resulting in the final concentrations of 31.25, 62.5, 125, 250 and 500 µg/ml. The inoculated wells were incubated at 37°C and the absorbance results were recorded at 600 nm after 24 h using VersaMax ELISA microplate reader (Molecular Devices). The minimum concentration of the compound that showed no turbidity visually as well as absorbance was recorded as MIC. Ampicillin was used as positive control in the final concentrations of 0.0625, 0.125 and 0.250 µg/ml.

Determination of minimum listericidal concentration (MLC)

MIC was also determined for *L. monocytogenes* with narrow range concentrations of 4-allylbenzene-1,2-diol as 62.5, 125, 150, 175, 200, 225, 250 and 500 µg/ml and the absorbance results were recorded at 600 nm after 24 h incubation at 37°C. Simultaneously the MLC of the compound was ascertained for the estimation of listericidal activity by viable count method. The MLC is defined as the lowest concentration required for killing 99.9% of the final inoculum after suitable incubation conditions. The survivability of the cells after broth microdilution treated with the compound was confirmed in TSA medium and the results were expressed as log CFU/ml. The culture in TSB medium was provided as control and compound with medium as

negative control. Ampicillin was used as a standard positive control and its MLC also determined by using 0.0625, 0.125, 0.25, 0.5 and 1.0 µg/ml of concentrations.

Time- dependent kill studies

Time-kill study is an important stricture to review the performance of antimicrobial agents. An overnight fresh *L. monocytogenes* culture (10^7 CFU/ml) inoculated into TSB medium was used for the time-kill assay of bioactive compound and incubated at 37°C. After 2 h, purified compound at 250 µg/ml of the final concentration was added to the culture. The optical density of the cells was measured at 600 nm for every 2 h up to 8 h. The viability of the cells was also assessed after 6 h of compound addition and articulated as log CFU/ml. In general, the bactericidal effect is attained with 90% kill for 6 h which is comparable to 99.9% for 24 h. The culture in the medium without compound considered as control whereas 70% ethanol as negative control.

Effect of 4-allylbenzene-1,2-diol on L. monocytogenes cell membrane

The cell wall damage on target cells caused by phenolic compound of betel leaves was analyzed by micrographs of High Resolution Scanning Electron Microscope (HRSEM). A fresh log growth phase Listerial culture in TSB medium by cell density of about 10^8 CFU/ml was treated with MLC of 4-allylbenzene-1,2-diol (250 µg/ml) for 4 h at 37°C and the untreated cells were served as control. The cells were centrifuged, washed with PBS buffer at pH-7.0 and fixed with 2.5% glutaraldehyde (Himedia) as primary fixative for 30 min. Then the washed cells were again post-fixed with 1% osmium tetroxide (Sigma) fixative for 30 min and dehydrated with sequence of gradient ethanol (25-100%). The air dried cells were placed on a stub then stammer-coated with gold and observed under F E I Quanta FEG 200- HRSEM.

Inhibition of biofilm formation

Effect of 4-allylbenzene-1,2-diol on biofilm formation of *L. monocytogenes* was checked using broth microdilution method. Serially two-fold diluted test samples (1X to 0.25X MIC) were prepared in 96-well microtitre plate and 12-well plate containing sterile glass cover slips at the final concentration ranged from sub-MIC 62.5 to MIC 250 µg/ml. A fresh log growth phase culture was serially diluted in TSB medium to attain 10^6 CFU/ml and the volume of 100 µl suspension was inoculated into each well. The compound and cells free medium were used as controls. After 48 h incubation, the cell turbidity was measured at OD_{570} nm using microplate reader. The supernatant from each well was discarded and washed thrice by using sterile PBS,

then the biofilms were stained with MTT dye to determine the viability of bacteria by observing OD values at 570 nm. The minimum biofilm inhibitory concentration (MBIC) was defined as the least concentration required to inhibit 90% of biofilm formation. Biofilm formation on glass cover slips by *Listeria* was also analyzed by acridine orange staining and fluorescence imaging.

SUPPLEMENTARY FIGURES

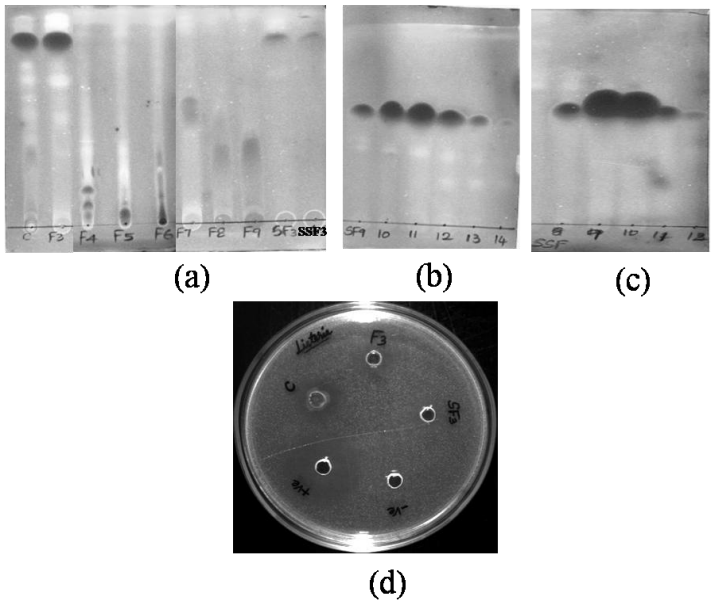

Figure S1. TLC profile and anti-*Listeria* activity of fractionated ethanol extract

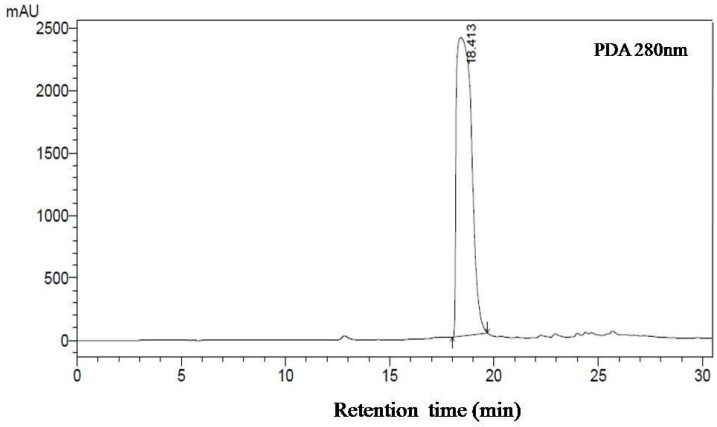

Figure S2. HPLC profile of bioactive constituent isolated from Pachaikodi variety of betel leaves

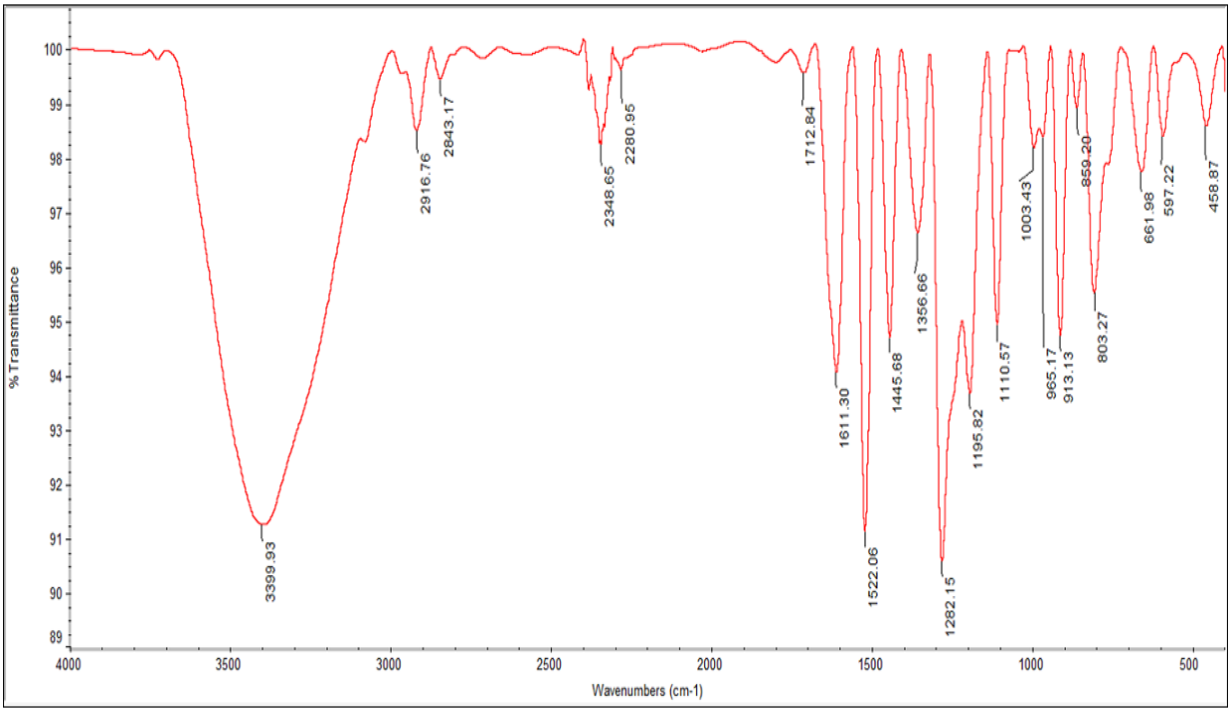

Figure S3. FT-IR spectrum of bioactive compound

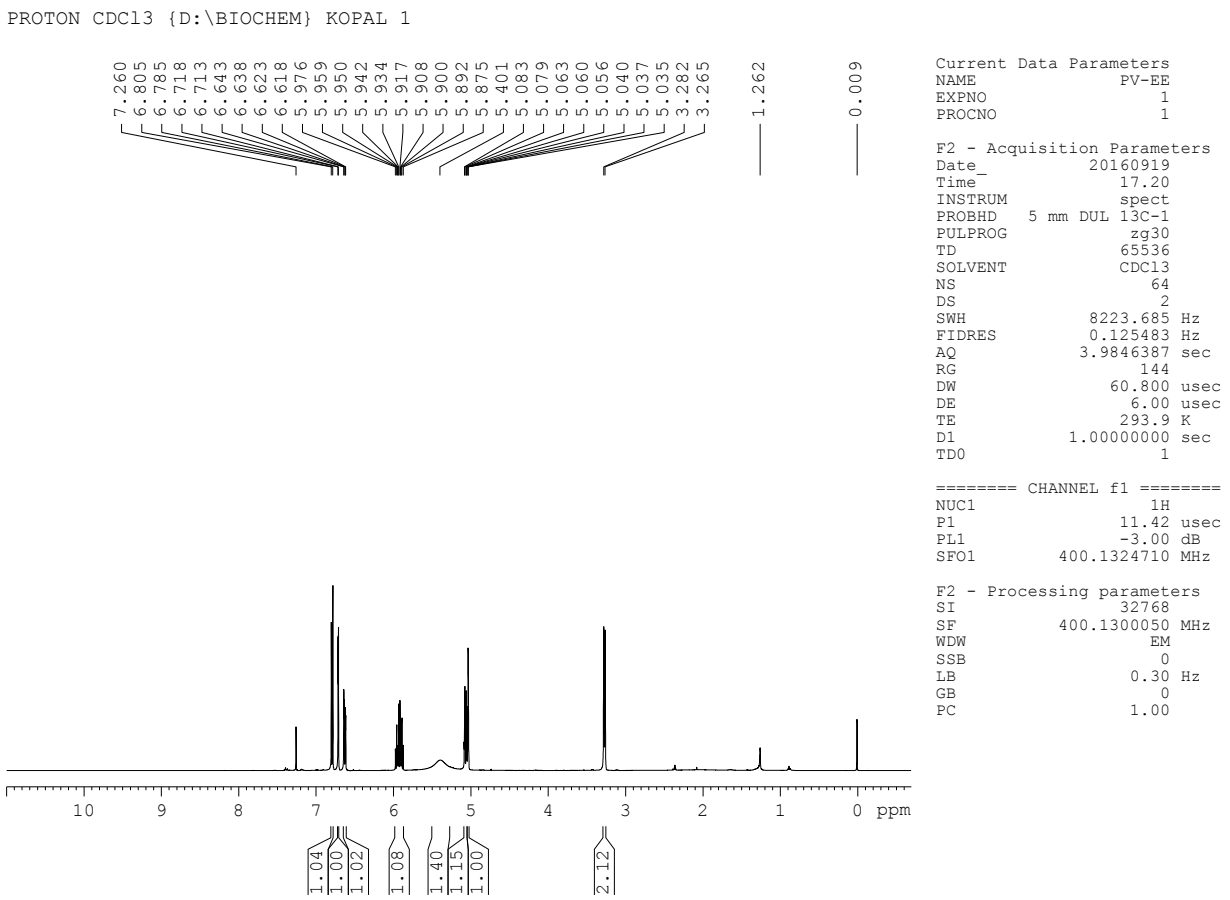


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Figure S4a. 1H-NMR spectrum of bioactive compound

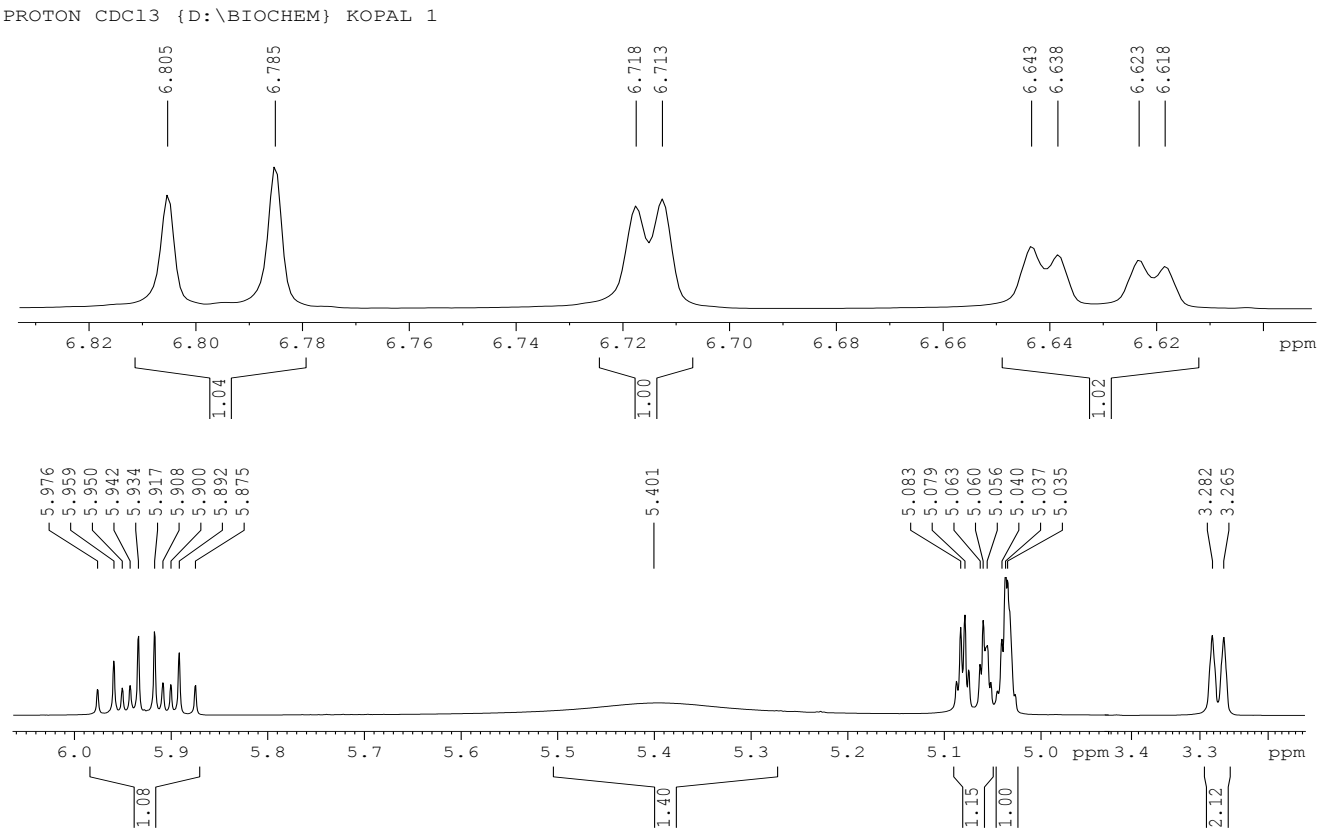

Figure S4b. Expanded 1H-NMR spectrum of bioactive compound

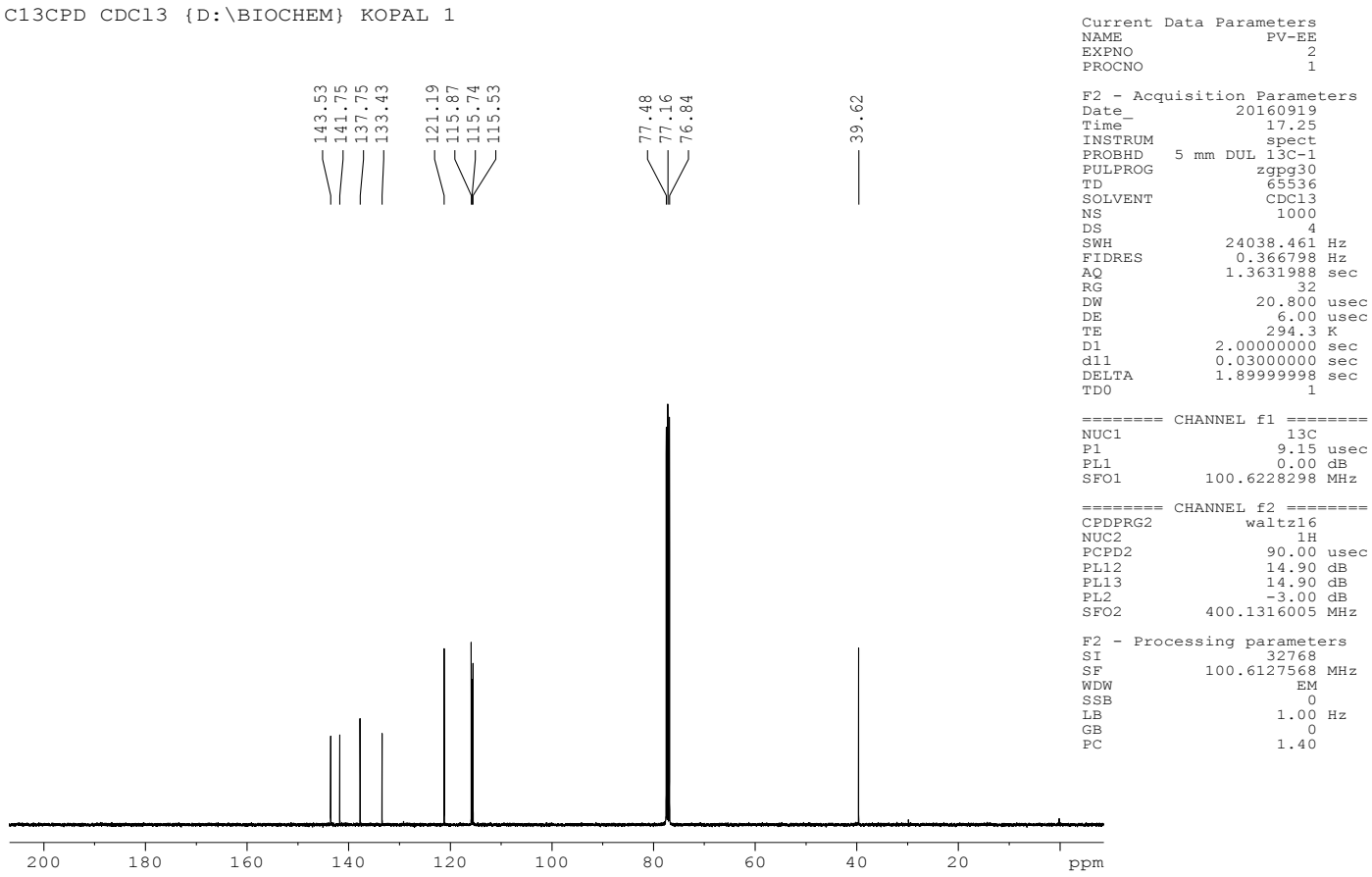

Figure S5. 13C-NMR spectrum of bioactive compound

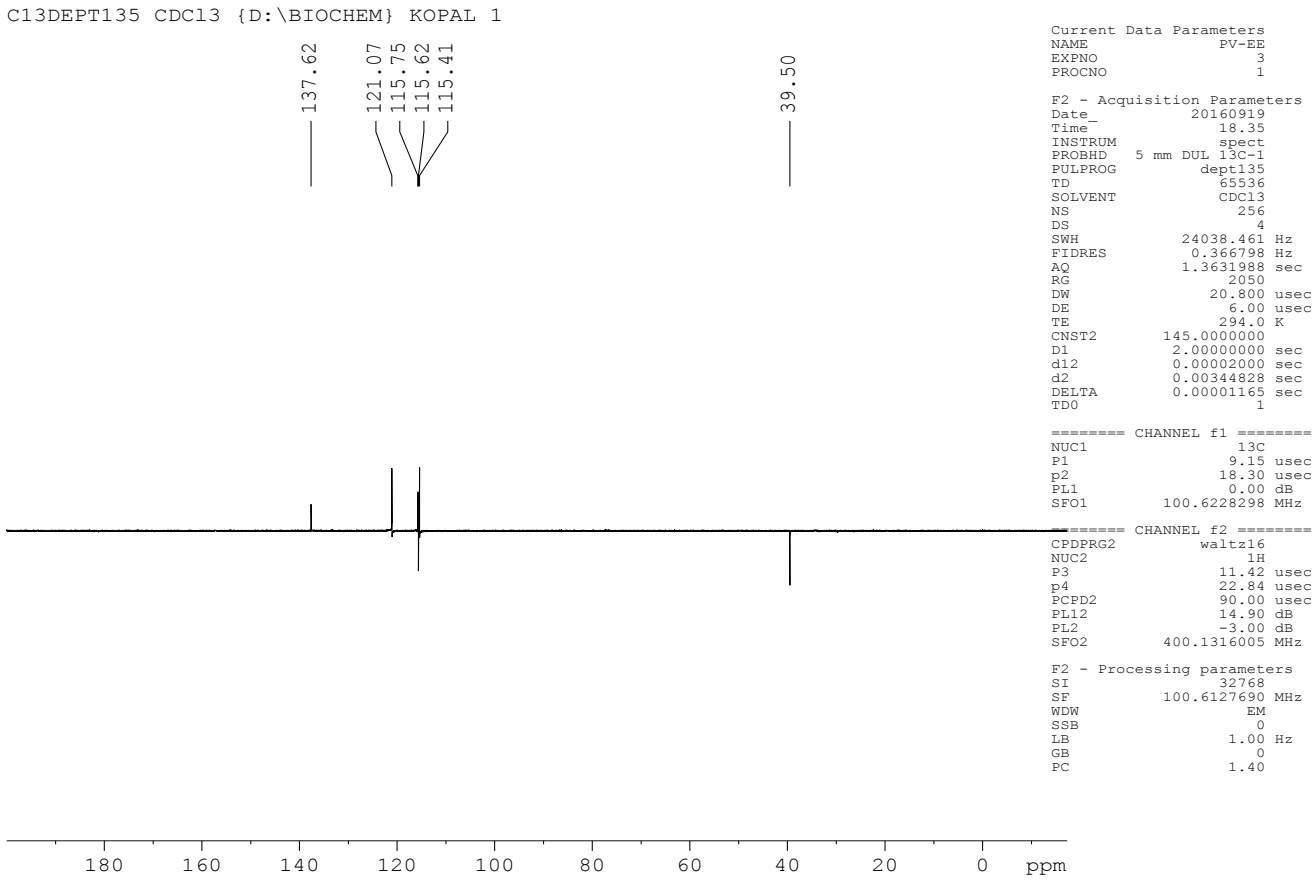

Figure S6. 13C DEPT135-NMR spectrum of bioactive compound

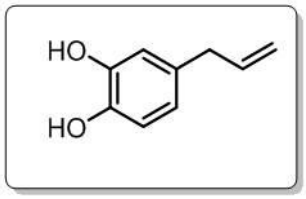

Figure S7. Structure of 4-allylbenzene-1,2-diol identified in ethanolic extract of Pachaikodi variety *P. betle* leaves

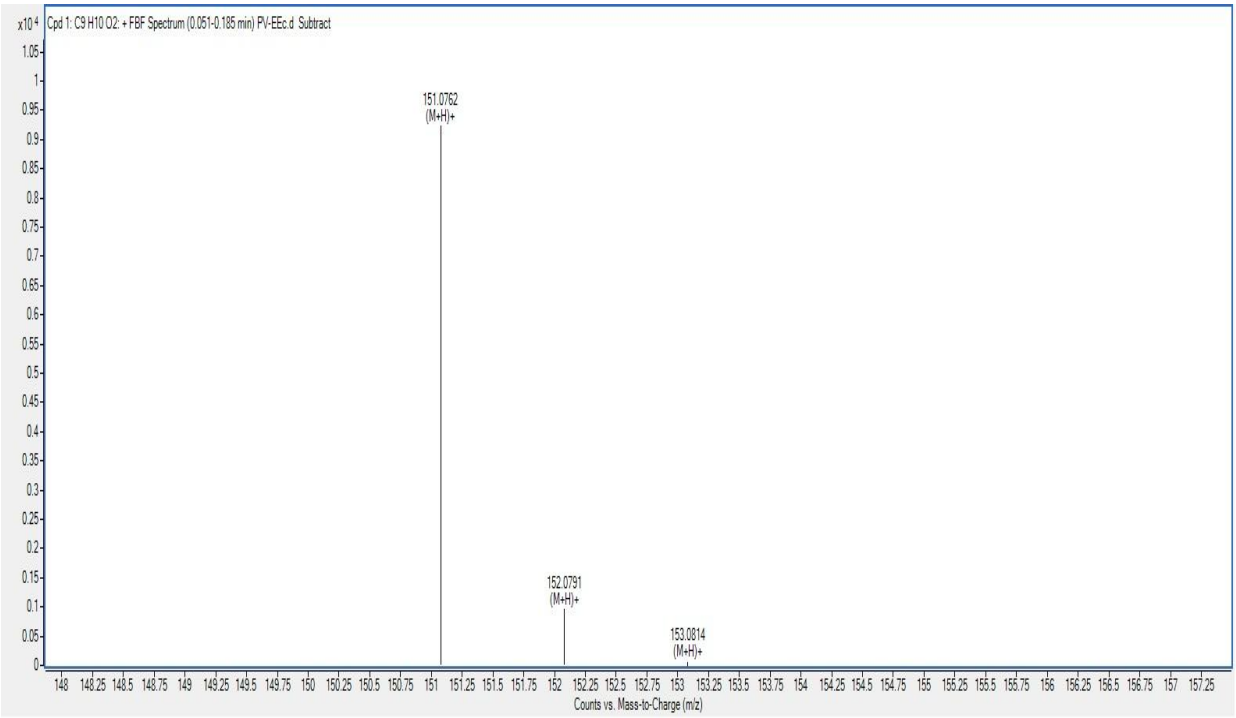

Figure S8. HRMS-ESI spectrum of bioactive molecule

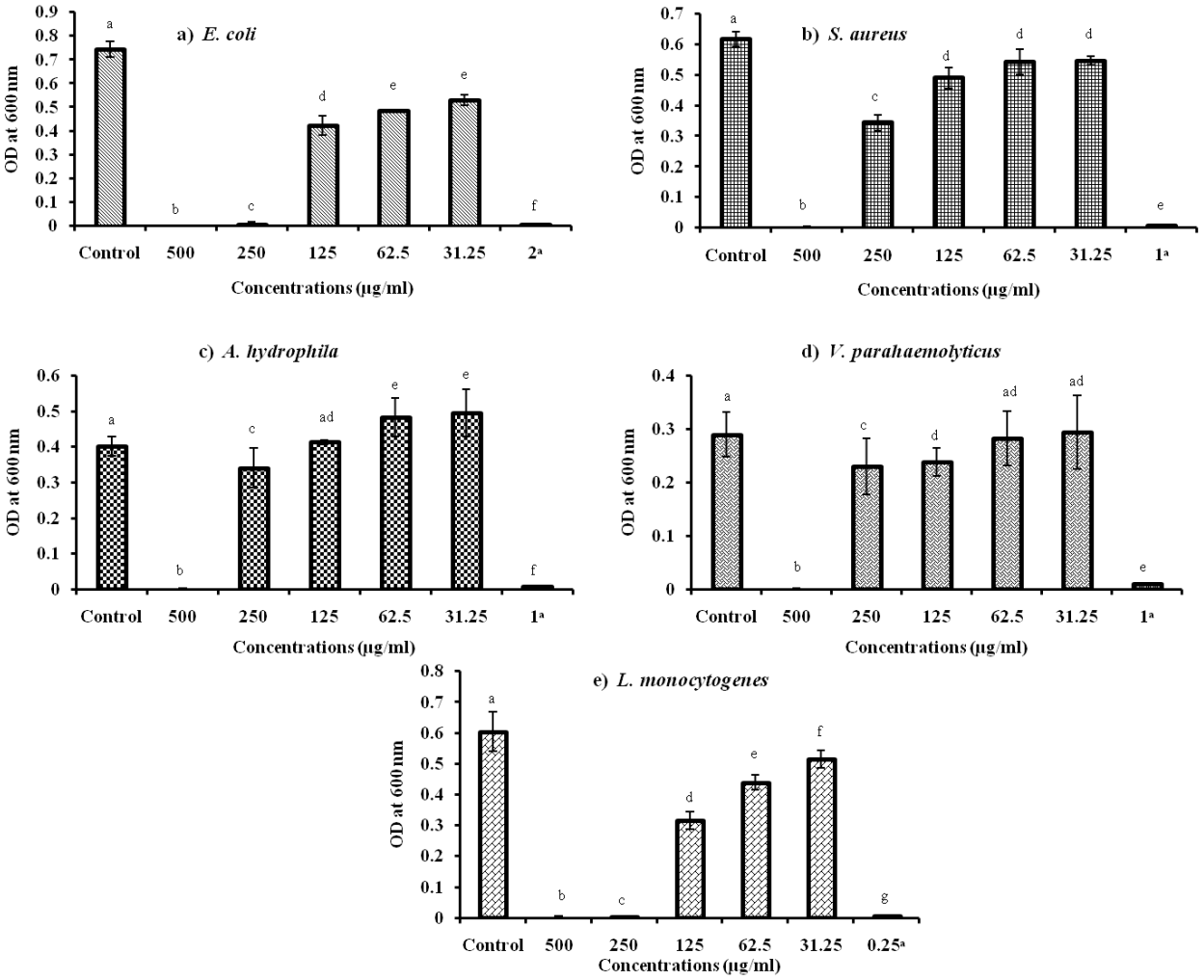

Figure S9. MIC of 4-allylbenzene-1,2-diol against different food borne pathogens.
The absorbance values are expressed as mean ± standard errors. The alphabets [a-g] indicate significant differences ($p < 0.05$) among various concentrations of compound when compared with control, however same alphabets indicate that there was no significant difference between the concerned groups. Whereas 0.25^a-2^a indicates the different concentrations of Ampicillin used as standard control.

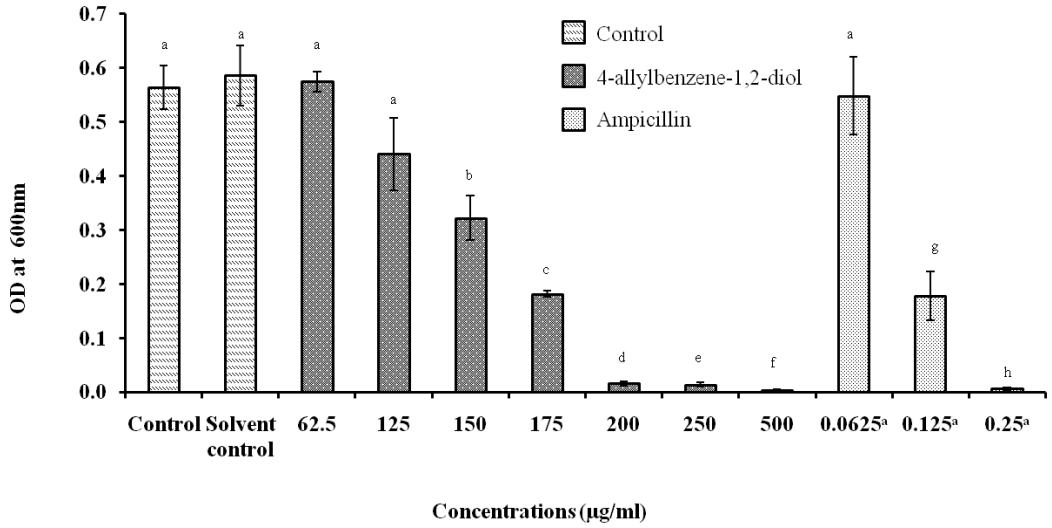

Figure S10. MIC of 4-allylbenzene-1,2-diol against *L. monocytogenes*.

The absorbance values are expressed as mean ± standard errors. The alphabets [a-h] indicate significant differences ($p < 0.05$) among various concentrations of compound when compared with control, however same alphabets indicate that there was no significant difference between the concerned groups. Whereas 0.0625^a - 0.25^a indicates the different concentrations of Ampicillin used as standard control.

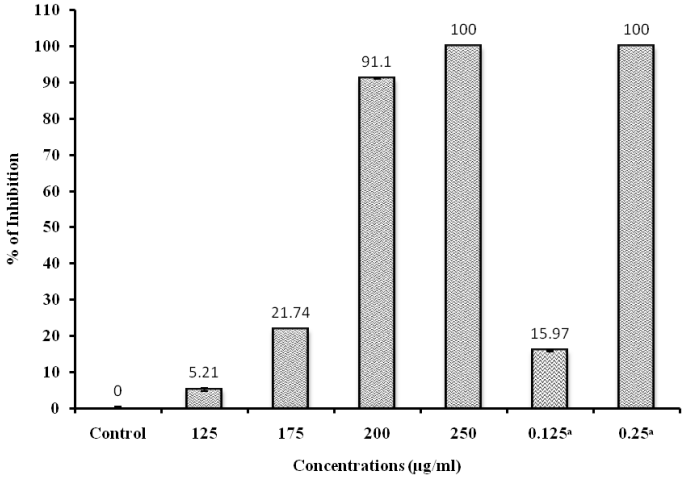

Figure S11. MLC of 4-allylbenzene-1,2-diol against *L. monocytogenes*.

The percentage values are expressed as mean ± standard errors. $0.125 – 0.25^a$ indicates different concentrations of Ampicillin as standard positive control.

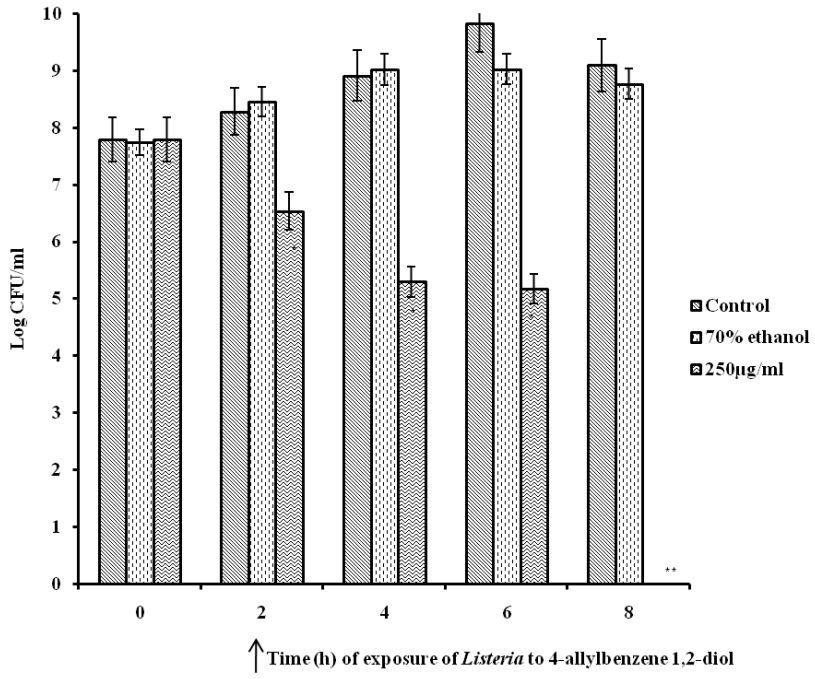

Figure S12. Time-kill assay of 4-allylbenzene-1,2-diol against *Listeria.*

The Log values are expressed as mean ± standard errors. * indicates significant level of difference at $p < 0.05$, whereas ** indicates significant level at $p < 0.01$ compared with control. The arrow indicates the time point in hours when 4-allylbenzene-1,2-diol was added for the treatment.

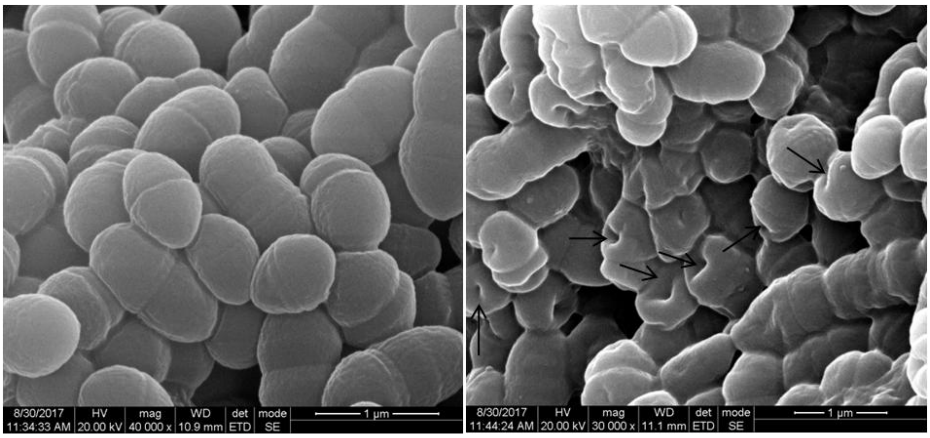

Figure S13. Effect of 4-allylbenzene-1,2-diol on the cell surface morphology of *L. monocytogenes* after 4 h treatment by SEM analysis.

The arrows show pore formation on the cell surface. Some deformity of cell morphology is also observed after treatment.

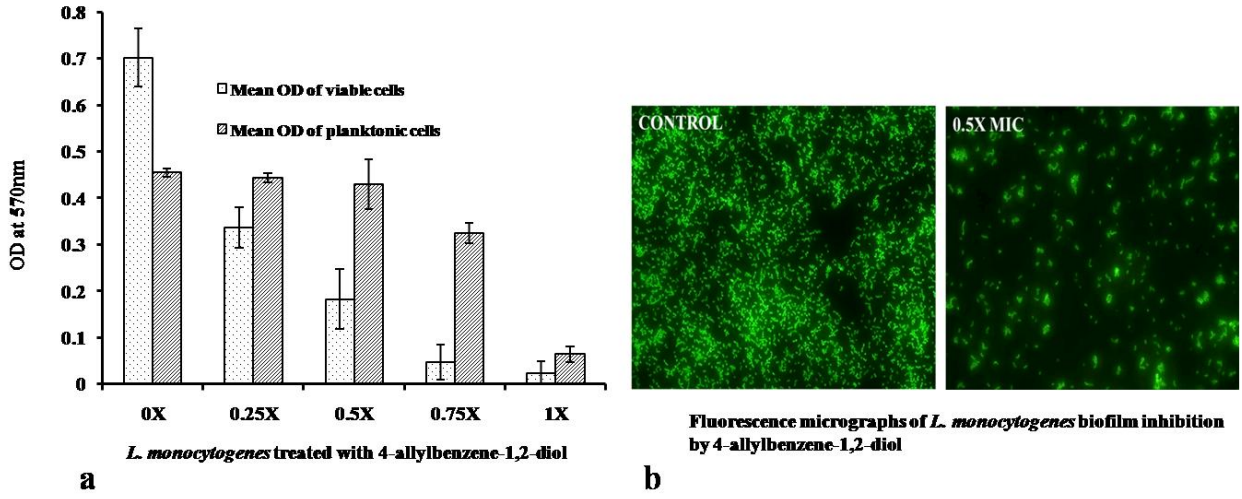

Figure S14(a). Effect of sub-MIC of 4-allylbenzene-1,2-diol against *L. monocytogenes* biofilm formation as measured by MTT cell viability assay.

The absorbance values are expressed as mean ± standard errors. 0.25X – 1X indicates different concentrations of 4-allylbenzene-1,2-diol ranging from 62.5 to 250 µg/ml.

Figure S14(b). Fluorescence micrographs of *L. monocytogenes* biofilm inhibition on sterile glass cover slip.

Biofilm developed during incubation at 37° C for 48 h in the presence and absence of 4-allylbenzene-1,2-diol (0.5X MIC).