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Proteomic Analysis and Antibacterial Effects of Lithospermi Radix against Common Bacteria from Human Infected Wounds

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Abstract:	The prevention and treatment of infections of human wounds are an important issue. In this investigation five herbal plants were selected, and their antibacterial effects were elucidated. Of these five herbal plants, the morphological changes of the one with the strongest antibacterial effects were observed using a scanning electron microscope (SEM) and the differential expression of bacterial proteins treated with them was determined by two-dimensional gel electrophoresis (2D-GE). Two proteins of weakly expressed spots from the highest matching percentage of 2D-GE were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF). The results indicated the Lithospermi radix have the best minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The SEM examination reveals different bactericidal morphological changes and colony distributions obtained using Lithospermi radix. 2D-GE reveals that Staphylococcus aureus treated with Lithospermi radix has the highest matching percentage (90.93%). Then identification of two proteins that are weakly expressed in Staphylococcus aureus by MALDI-TOF/TOF reveals that they are Holliday junction ATP-dependent DNA helicase RuvB and D-alanine:D-alanine ligase (DDI). These results are noteworthy to suggest that Lithospermi radix may be used as an antibacterial medicine to treatment of human wound infection.
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1 Proteomic Analysis and Antibacterial Effects of *Lithospermi*
2 *Radix* against Common Bacteria from Human Infected Wounds

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19

Abstract

The prevention and treatment of infections of human wounds are an important issue. In this investigation five herbal plants were selected, and their antibacterial effects were elucidated. Of these five herbal plants, the morphological changes of the one with the strongest antibacterial effects were observed using a scanning electron microscope (SEM) and the differential expression of bacterial proteins treated with them was determined by two-dimensional gel electrophoresis (2D-GE). Two proteins of weakly expressed spots from the highest matching percentage of 2D-GE were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF). The results indicated the *Lithospermi radix* have the best minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The SEM examination reveals different bactericidal morphological changes and colony distributions obtained using *Lithospermi radix*. 2D-GE reveals that Staphylococcus aureus treated with *Lithospermi radix* has the highest matching percentage (90.93%). Then identification of two proteins that are weakly expressed in Staphylococcus aureus by MALDI-TOF/TOF reveals that they are Holliday junction ATP-dependent DNA helicase RuvB and D-alanine:D-alanine ligase (DDI). These results are noteworthy to suggest that *Lithospermi radix* may be used as an antibacterial medicine to treatment of human wound infection.

39 *Keywords:* Wound infection; Antibacterial effects; Herbal plant; Mass spectrometry;

40 Scanning electron microscope

1. Introduction

All wounds contain bacteria and a limited amount of bacteria will be present even if a wound is healing normally. However, if the number of bacteria rises, the wound may become infected. Bacterial overload in a wound can cause a serious infection that requires antibiotic or surgical treatment. A survey sponsored by the World Health Organization demonstrated a prevalence of nosocomial infections from 3 to 21%, 5-34% of which are caused by wound infections.[1,2] Accordingly, the prevention of wound infection is important. Generally, the use of antibiotics is the most simple and common method of treating or preventing wound infection. However, the emergence of antibiotic resistant bacterial strains is a growing problem,[3,4] which has led to an urgent global call for new antimicrobial drugs, particularly from natural sources. The use of herbal medicines in the US has increased by 20% annually since the 1990s and the sale of non-prescribed herbal medicines is estimated to be US$ 4 billion per year.[5] The bacteria that are most commonly isolated from infected wound include *Staphylococcus aureu*s, Coagulase-negative *staphylococci*, *enterococci*, *Escherichia. coli* and *Pseudomonas aeruginosa*.[6,7] Since *S. aureus* and *E. coli* are the most common gram-positive and gram-negative bacteria that cause wound infection and *P. aeruginosa* is an important nosocomial and highly prevalent opportunistic pathogen,[8,9] these three bacteria are selected as the targets in the present study. Based on previous

60 studies,[5,10-15] five herbal plants (*Aloe barbadensis, Mentha piperta, Lithospermi radix,*

61 *Callicarpa japonica* and *Forsythia suspensa*) were selected to evaluate their

62 antibacterial activities toward to *S. aureus*, *E. coli* and *P. aeruginosa*. The

63 morphological changes and colony distributions of the tested bacteria were examined

64 using a scanning electron microscope (SEM). The differential expressions of the

65 proteins are compared using two-dimensional gel electrophoresis (2D-GE); the

66 proteins with the best matching results are identified by matrix-assisted laser

67 desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF) and their

68 functions are evaluated.

69

70 # 2. Materials and Methods

71 ## 2.1 Preparation of plant extracts

72 The five selected plant samples that were adopted herein were purchased in January
73 2009 from a local market. Parts of the plants (whole plant, bark, root, leaves, tubers
74 and a mixture of these) were chopped into small pieces, dried in air at room
75 temperature (25 °C) in the shade and pulverized using a laboratory mill, yielding
76 300–400 g. The fine powders were separately soaked in 75% methanol (3 × 500 mL)
77 for four hours at room temperature to produce extracts. The extracts were filtered and
78 concentrated using a rotary evaporator at 50 °C and 100 mbar. Further steps in the
79 preparation were taken from a method described in a previous study.[16] The extracts
80 were transferred into freeze-drying equipment (Freeze dryer FDU-1200)

81

82 ## 2.2 Bacterial strains and cultures

83 Reference isolates *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), and *P.*
84 *aeruginosa* (ATCC 27853) were obtained from the Food Industry Research and
85 Development Institute of Taiwan. Luria-Bertani medium (LB) were used for
86 propagation and maintenance of bacterial cultures. Broth cultures were incubated
87 under aerobic conditions at 37℃, whereas solid cultures were incubated at 37℃ and
88 5% CO_2.

89 **2.3 Evaluation of antibacterial activity**

90 The antibacterial activity of the extracts was evaluated using a micro-dilution

91 bioassay in 96-well micro-plates. For each, minimum inhibitory concentration (MIC)

92 was determined. The broth dilution method was used to determine the MIC of

93 reference bacterial strains.[17-20] The bacterial strains were cultured overnight with

94 incubation at 37 °C (FIRSTEK MODEL: S300). The test strains were suspended in

95 sterile physiological saline to yield a final density of 1×10^6 cfu/mL. A serial of

96 two-fold diluted plant extracts was obtained using Muller Hinton broth (BBL™

97 Muller Hinton Broth). A microtiter plate was set up and an extract of the herbal plants

98 at a starting concentration of 50 mg/ml was transferred into the first well. Serial

99 dilutions were conducted to yield concentrations of 25, 12.5, 6.4, 3.2, 1.6, 0.8, 0.4 and

100 0.2 mg/ml; the last well contained Muller Hinton broth without extract as a negative

101 control. An inoculum of a prepared bacteria culture with approximately 1×10^6 cells

102 was added to each of the wells and incubated at 37°C for 24 h. The MIC was taken as

103 the lowest concentration that inhibited any visible bacterial growth on the culture

104 plates.[21] 10 μL from each tube of MIC broth with visible growth was even deposited

105 onto nutrient agar plates. Following overnight incubation, the plates were examined

106 for colony growth. Lack of growth indicated that the tested drug was bactericidal,

107 while growth indicated that the drug was bacteriostatic at the relevant dilution. To

108 establish minimum bactericidal concentration (MBC), 10 μL of each culture medium
109 without visible growth was used. After 16-24 h of aerobic incubation at 35 °C, the
110 surviving organisms were counted. The MBC was the lowest concentration at which
111 99.9% of the bacteria are killed.
112
113 **2.4 Scanning electron microscopy (SEM)**
114 The bacteria that were susceptible to the plant extracts were prepared for
115 examination using a SEM. Samples were prepared as described elsewhere.[22] The
116 samples were fixed in 2.5% glutaraldehyde in 0.1 M Tris buffer (pH 7.3) at 4°C for 1
117 hr. After they had been washed twice in a Tris buffer with 5% sucrose, they were
118 post-fixed in 1.0% osmium- tetroxide solution in the same buffer for 1 hr. The
119 resulting osmium-treated samples were then washed with 0.1 M Tris buffer and
120 dehydrated by successive extractions with 50, 70, 80, 90 and 95% ethanol, each for a
121 period of 10 min, and finally with 100% ethanol for 15 min twice. They were then
122 dried using the CO_2 critical-point drying technique, coated with gold and examined
123 using a SEM (Hitachi S-3000N, Japan).
124
125 **2.5 Two-dimensional gel electrophoresis (2D-GE) and MALDI-TOF/TOF MS**
126 Bacteria were grown on Luria-Bertani medium (LB) and maintained at 37°C. They

127 were collected for sonication on ice using a microtip with the power level set between
128 4 and 5 at 20% duty, with a 10s short burst followed by a 10s interval, repeated for 20
129 minutes. The total proteins were precipitated using 10% trichloroacetic acid (TCA)
130 and were separated by 2D-GE, as follows. The protein sample (600μg) was loaded
131 onto 18cm-long Readystrip IPG strips (Amersham Biosciences, UK) at a pH range of
132 3–10NL (nonlinear) and layered with 0.8 ml of covering oil to prevent drying of the
133 gel or crystallization of the urea. The gel was then run on an Ettan IPGPhor II
134 (Amersham Biosciences, UK) at 30 V to rehydrate the gel strip for 16 h; then
135 programs with 500 V for 1 h (500 Vh), 1000 V for 1 h (1000 Vh), and 8000 V for 8 h
136 (64000 Vh) were run. The voltage ramped automatically owing to the increase in
137 resistance of the strip as excess ions exited it. After the first-dimension IEF, the strip
138 was washed to remove the cover oil and then equilibrated for 12~15 min in 5 ml of
139 equilibration buffer, which contained 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS,
140 30% glycerol, and 1% DTT. The strip was then subjected to a second equilibration,
141 using 5 ml equilibration buffer, which was like the first, but with the DTT replaced by
142 1.5% iodoacetamide, for an additional 12~15 min. Next, SDS-PAGE was run using a
143 PROTEAN II xi cell tank (Bio-Rad, USA) at 200 V for 4 h. The results were further
144 analyzed by calculating software (Image Master 2D Platinum, GE Healthcare).
145 Following electrophoresis, the gel was stained with 0.25% (w/v) coomassie R-250

146 (Amersham Biosciences, UK), and spots that revealed variation of expression among
147 strains were selected manually and digested by trypsin for subsequent
148 MALDI-TOF/TOF analysis. Mass spectrometry (MS) and protein identification were
149 conducted by Mission Biotech Co., Ltd., Taiwan. Tandem MS was carried out on a
150 QSTARXL (Applied Biosystems-Sciex, Ontario, Canada) hybrid
151 quadropole-time-of-flight mass spectrometer. Mascot software (Matrix Sciences Inc.,
152 Beachwood, OH, USA) was used to identify proteins against the NCBInr protein
153 database of the National Center for Biotechnology Information at the National
154 Institutes of Health.
155

156 # 3. Results

157 ## 3.1 The antimicrobial activities of plant extracts.

158 The MICs and MBCs of the five herbal plants (*Aloe barbadensis, Mentha piperta,*
159 *Lithospermi radix, Callicarpa japonica* and *Forsythia suspensa)* against *S. aureus*, *E.*
160 *coli* and *P. aeruginosa*, were determined (Table 1). Of these five herbal plants,
161 *Lithospermi radix* exhibit potent antibacterial activities. Accordingly, *Lithospermi*
162 *radix* was used in further tests.

163

164 ## 3.2 Preparation of samples and two-dimensional gel electrophoresis (2D-GE)

165 Changes in the extracellular proteins of the three aforementioned bacteria upon
166 exposed to *Lithospermi radix* were determined. Table 2 shows the final concentrations
167 used in 2D-GE. The capacity of *Lithospermi radix* to induce or suppress the
168 expression of various extracellular proteins was determined by examining the
169 resulting culture supernatants using 2D-GE and IEF from pH 3 to pH 10. Figures 1-3
170 present typical 2D gel images of each bacterium with/without exposure to *Lithospermi*
171 *radix*. The molecular weights of most expressed proteins are between 29 kDa to 97
172 kDa. Although the antibacterial effect of *Lithospermi radix* is evident, most proteins
173 are preserved and the matching percentage range is 79.98 % to 90.93 % (Table 3).
174 The increased spots are more than decreased spots in *S. aureus* and *P. aeruginosa*.

175 The increased spots are a little bit more than decreased spots while *E. coli* treating
176 with *Lithospermi radix*. The proteins of *S. aureus* and *P. aeruginosa* are acidic,
177 between pH 4 – 6, and the proteins of *E. coli* are mildly acidic, between pH 5 – 7.
178
179 **3.3 Scanning electron microscopy (SEM)**
180 SEM was adopted to observe the morphological changes to bacteria that were
181 treated with *Lithospermi radix*. A comparison of the cells of *S. aureus* that were
182 treated with *Lithospermi radix* to untreated cells revealed that treatment caused no
183 apparent morphological changes but did reduce the number of cells, spreading them
184 out (Fig. 4). The treated *E. coli* cells also exhibited no apparent morphological change
185 (Fig. 5), maintaining a regular rod shape, but their number and length decreased. In *P.*
186 *aeruginosa*, cells appeared to aggregate with multiple connected filaments and to
187 deform upon treatment with *Lithospermi radix* (Fig. 6).
188
189 **3.4 Proteomic analysis of *S. aureus* treated with *Lithospermi radix***
190 To identify changes to bacterial proteins that are caused by treatment with
191 *Lithospermi radix*, a proteomic analysis of *S. aureus* was performed. Candidate
192 proteins from decreased spots that are involved in the bactericidal mechanism of
193 *Lithospermi radix* were selected randomly. The protein profiles of *S. aureus* before

194 and after treatment with *Lithospermi radix* (Fig. 1) were compared. A total of 355 and

195 328 protein spots were detected by 2D-GE analysis before and after this treatment,

196 respectively, and the match percentage was 90.93 % (311 spots).

197 Those changes in protein expression upon treatment were detected and excisions

198 from gels were followed up by protein determination. Two proteins that were weakly

199 expressed in *S. aureus* after treatment of *Lithospermi radix* were subjected to

200 MALDI-TOF/TOF MS determination of proteins. They are Holliday junction

201 ATP-dependent DNA helicase RuvB and D-alanine:D-alanine ligase (DDl).

202

203 ## 4. Discussion

204 Antibiotics are the first choice for treating bacterial infection. Different antibiotics
205 should be used to treat different bacteria. No single antibiotics can treat a combined
206 infection of *S. aureus*, *E. coli* and *P. aeruginosa*. In such cases, doctors must prescribe
207 a combination of antibiotics to treat wound infections with these three bacteria
208 simultaneously before the results of a wound culture have bee obtained. Sometimes,
209 such infections not only require the use of expensive antibiotics but also increase
210 morbidity and mortality. Additionally, a greater period of exposure is associated with
211 greater risk of developing resistance, independently of the need for the antibiotics. As
212 antibiotic resistance becomes more common, the need for alternative treatments
213 increases. These involve other agents with greater antibacterial effect and lower
214 toxicity. New antibiotics are necessary to treat microbial pathogens that are becoming
215 increasingly resistant to available treatment. However despite a push for new
216 antibiotic therapies, the rate of approving drugs has been declining.[23]

217 Plants contain numerous antibacterial constituents that can be used for treatment in
218 cases of multiple bacterial infections. Of these five herbal plants in our study,
219 *Lithospermi radix* (the root of Lithospermum erythrorhizon Siebold. et Zuccarinii)
220 exhibit potent antibacterial activities. The main ingredients of *Lithospermi radix*
221 includes Shikonin , Isobutylshikonin , β-Hydroxy isovaleryl shikonin , α-Meth

222 yl-n-butylshikonin and quinoid. During these, Shikonin is the main ingredient of
223 antibacterial effect. *Lithospermi radix* extract can inhibit the release of histamine and
224 the production of inflammatory cytokine in mast cells and is utilized to treat such
225 conditions as eczema, skin burns and frostbite.[24]
226 The images obtained by SEM demonstrate that these three bacteria undergo
227 different morphologic changes and colony distribution upon treatment with
228 *Lithospermi radix,* indicating the different antibacterial mechanisms to the different
229 bacteria. Furthermore, even an individual herbal plant can have different antibacterial
230 mechanisms against different bacteria.
231 The bacteria reveal different morphologic changes and colony distribution after
232 treatment with *Lithospermi radix*. Even though the same herb plant, there are different
233 mechanisms of antibacterial effect.
234 RuvB is a hexameric ATPase. The RuvB ATPase provides the energy to drive the
235 exchange of base pairs that move the DNA branch in Holliday junction for DNA
236 homologous recombination.[25] D-alanine:D-alanine ligase (DDl) participates in
237 bacterial cell wall biosynthesis, making it a target for the development of new
238 antibiotics.[26,27] Accordingly, RuvB and DDl may be two important targets of the
239 bactericidal activity of *Lithospermi radix*, providing new insights into the
240 pharmaceutical value of *Lithospermi radix* in preventing bacteria infection.
241

242 ## 5. Conclusions

243 Studies on the antibacterial effect of plants are worth to be studied in the future.
244 The observed activity validates the plants used in traditional medicine. It is
245 noteworthy to suggest that *Lithospermi radix* may be used as an antibacterial
246 medicine to treatment of human wound infection.

247

248 ## Acknowledgements

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252

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327

328 ## Captions

329 Figure 1. 2D-GE analysis of total proteins in plant extracts treated *S. aureus.*

330 Figure 2. 2D-GE analysis of total proteins in plant extracts treated *E. coli.*

331 Figure 3. 2D-GE analysis of total proteins in plant extracts treated *P. aeruginosa.*

332 Figure 4. SEM images of *S. aureus* treated with plant extracts.

333 Figure 5. SEM images of *E. coli* treated with plant extracts.

334 Figure 6. SEM images of *P. aeruginosa* treated with plant extracts.

335

336 Table 1 The MIC and MBC of plant extracts against reference strains.

337 Table 2 The concentrations of plant extracts for treating bacteria in 2D-GE analysis.

338 Table 3 The summary of 2D-GE analysis in plant extracts treated bacteria.

Figure1
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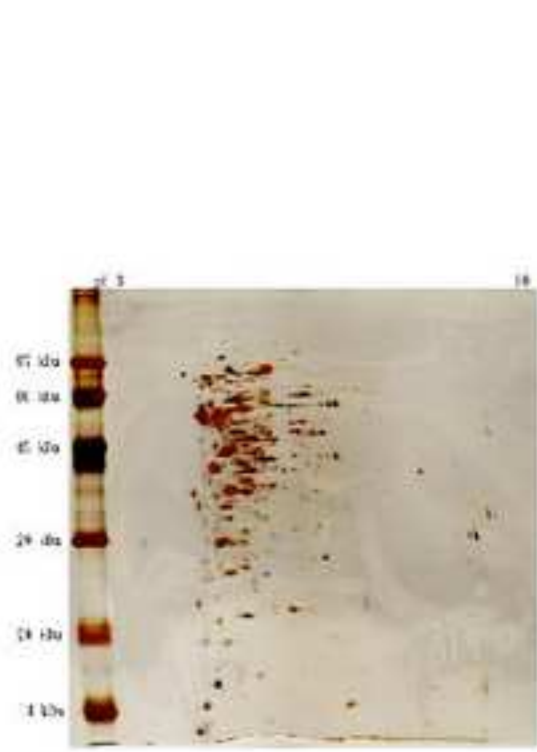
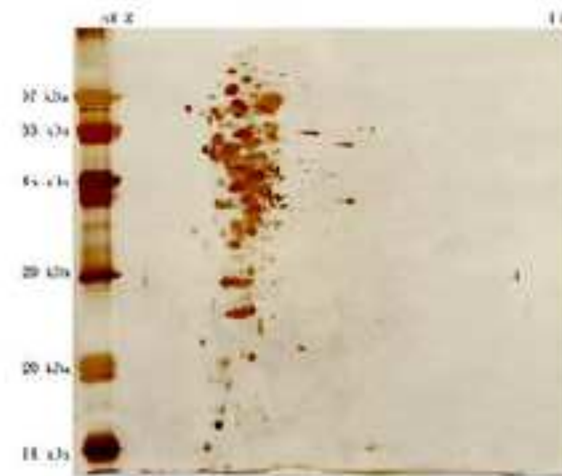
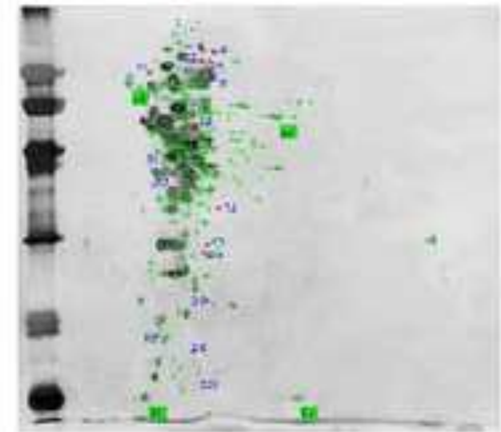
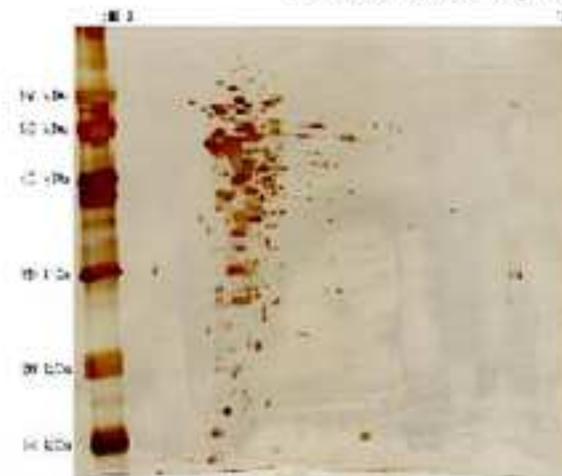


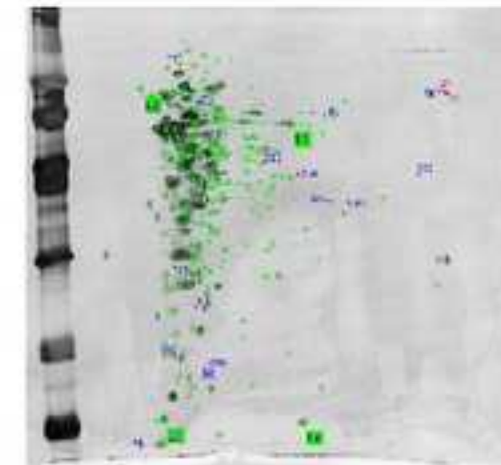
S.aureus





S.aureus (0.25 mg/ml *Aloe barbandensis*)





S.aureus (6.25 μg/ml *Lithospermi Radix*)

Figure2

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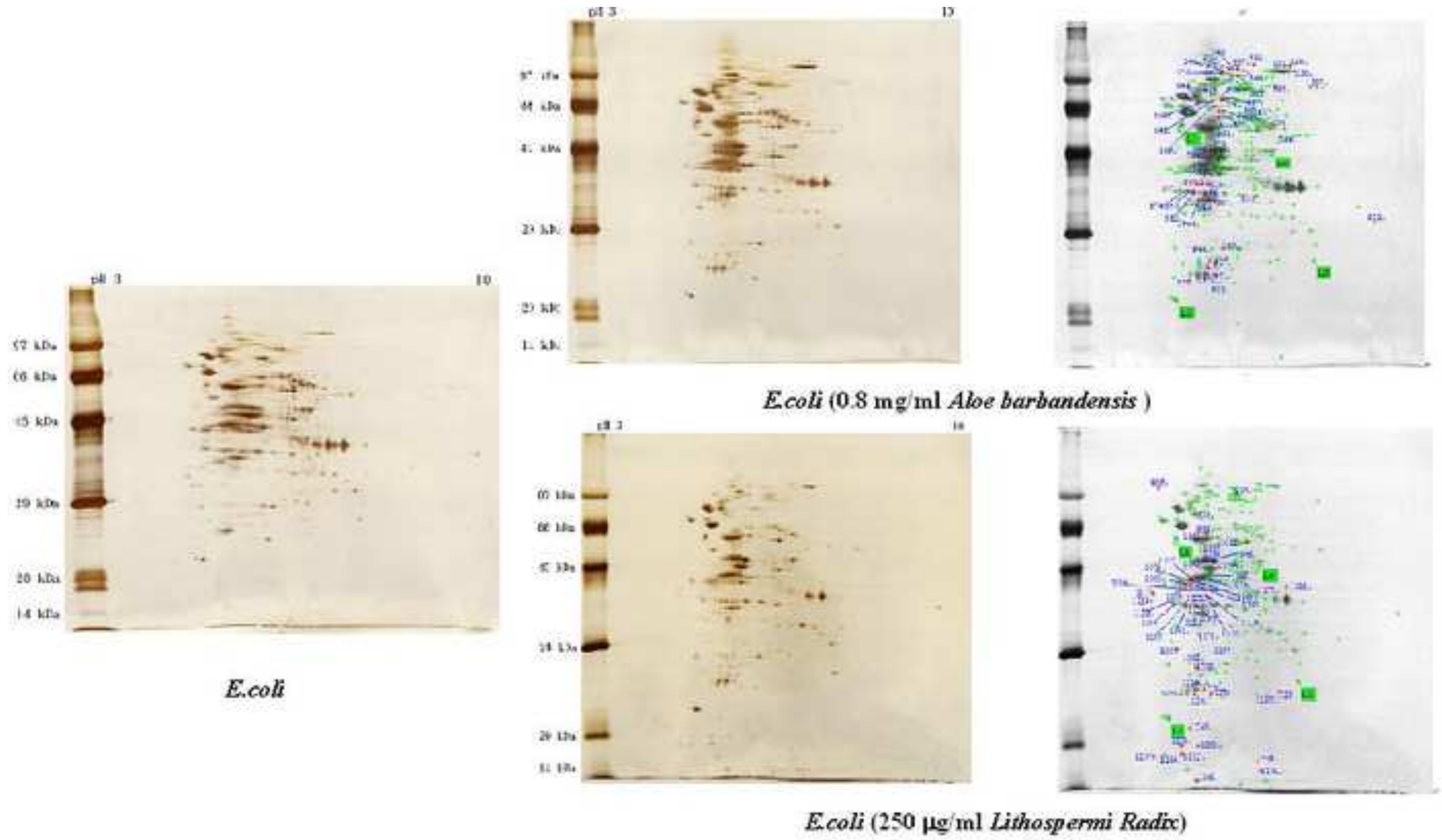

Figure3
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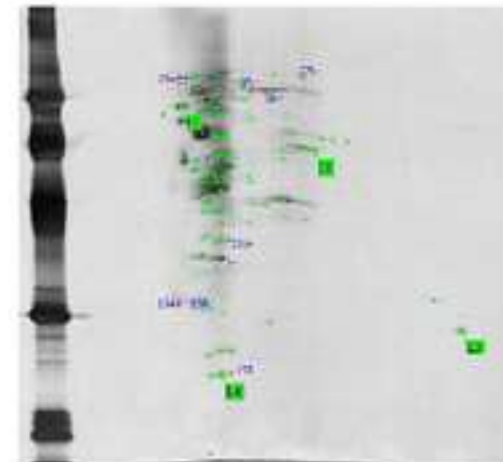
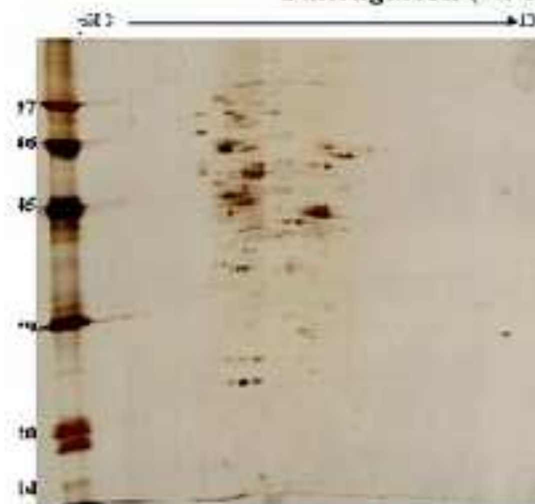
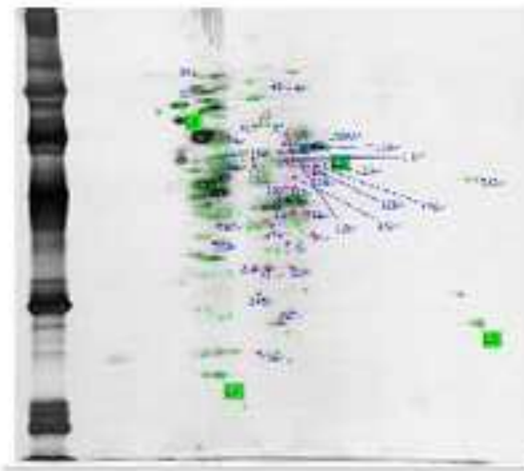
P.aeruginosa



P.aeruginosa (0.8 mg/ml *Aloe barbandensis*)





P.aeruginosa (0.1 mg/ml *Lithospermi Radix*)

Figure4
Common.Links.ClickHereToDownloadHighResolutionImage

S.aureus

+ *Lithospermi radix* (6.25 μg/ml)

+ *Aloe barbadensis* (0.25 mg/ml)

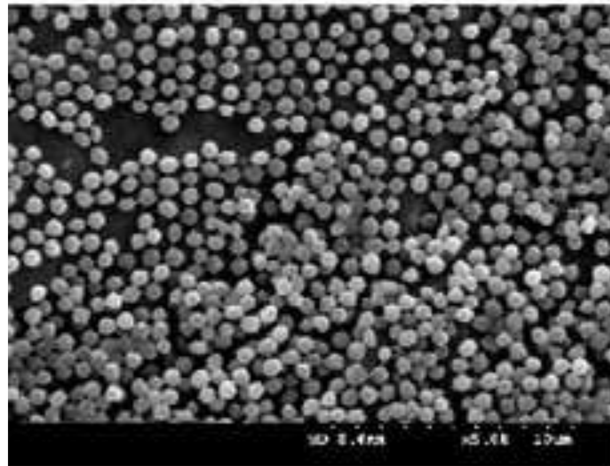
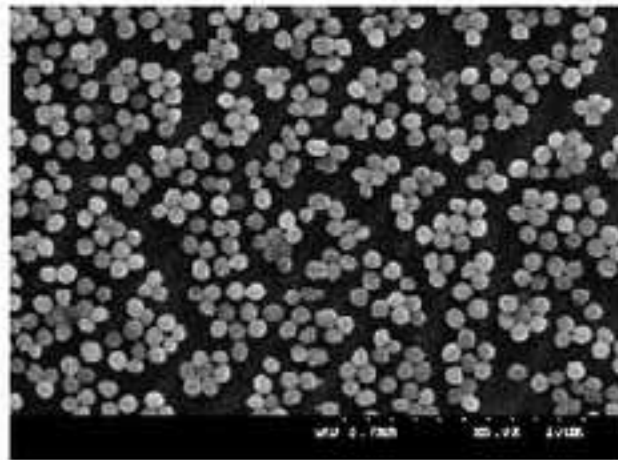
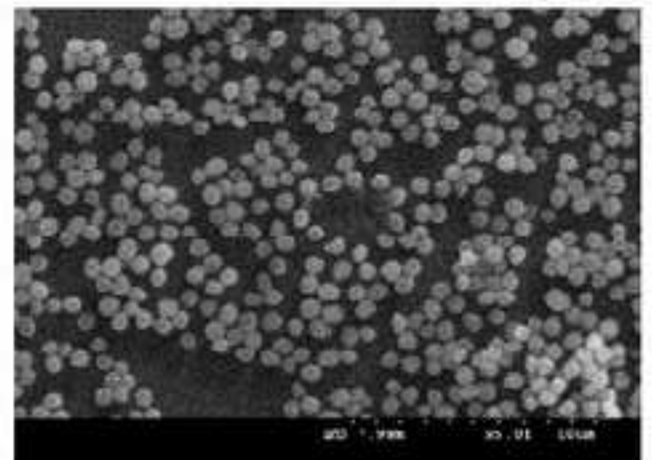
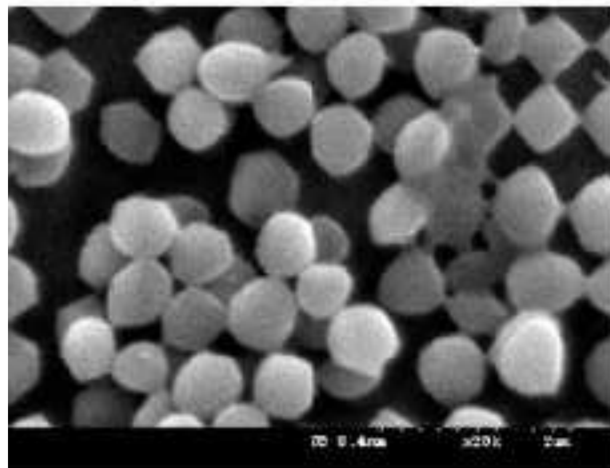
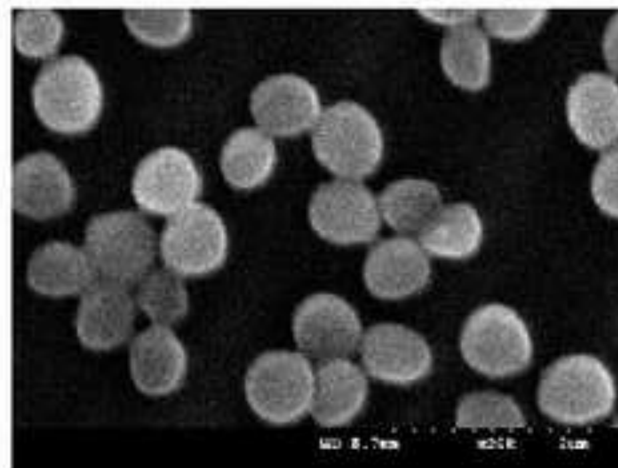
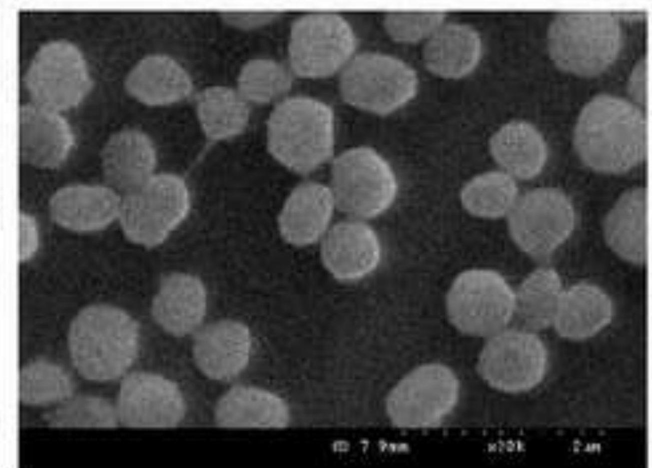







Figure5
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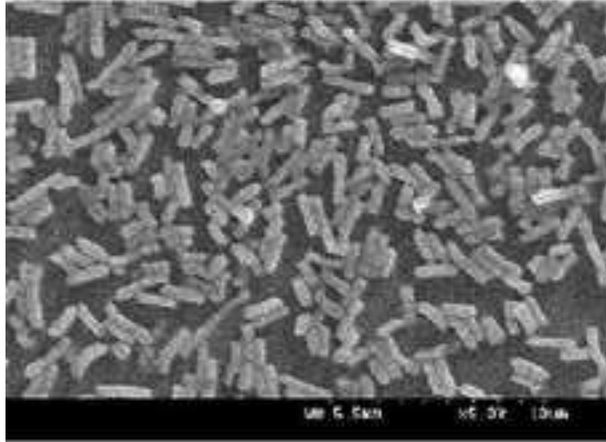

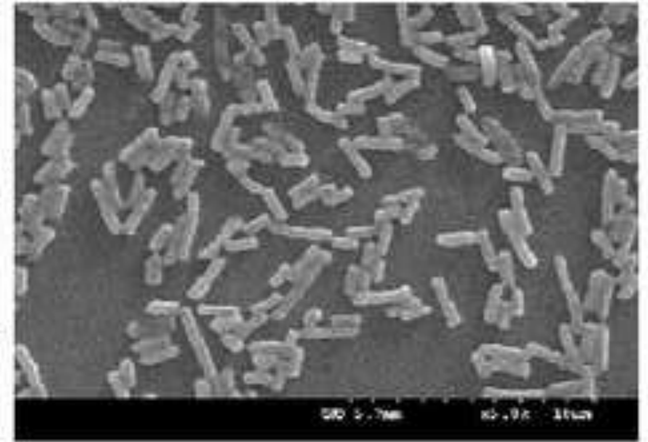

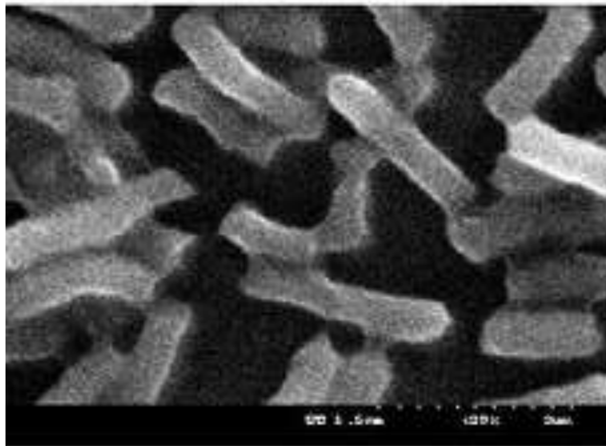

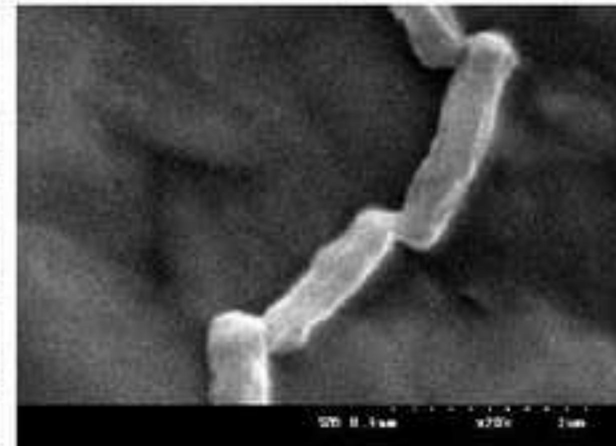

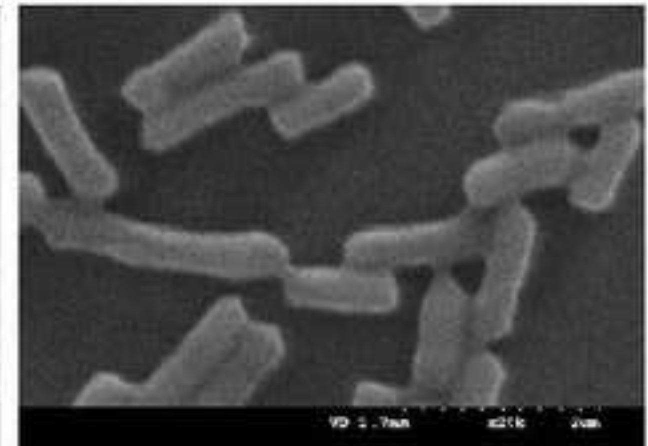

Figure6

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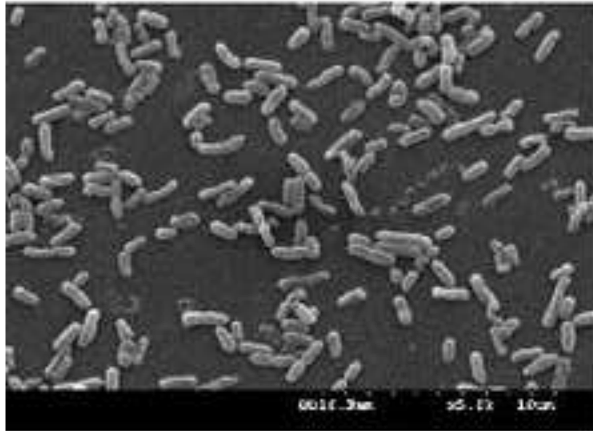
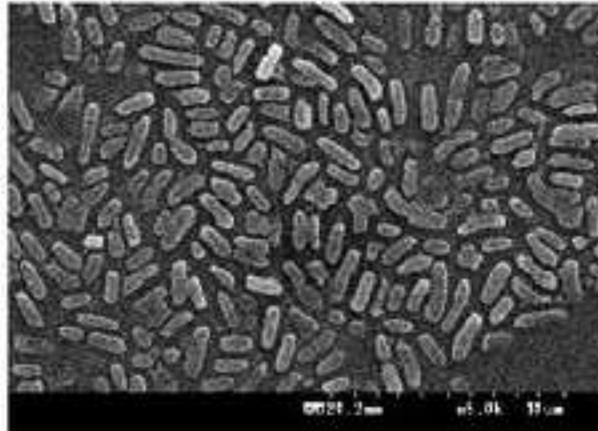
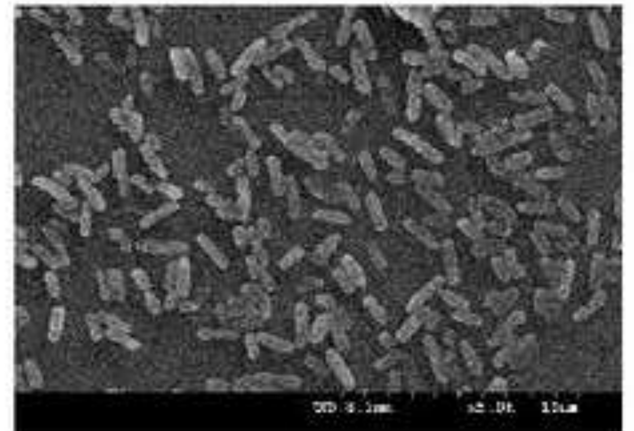
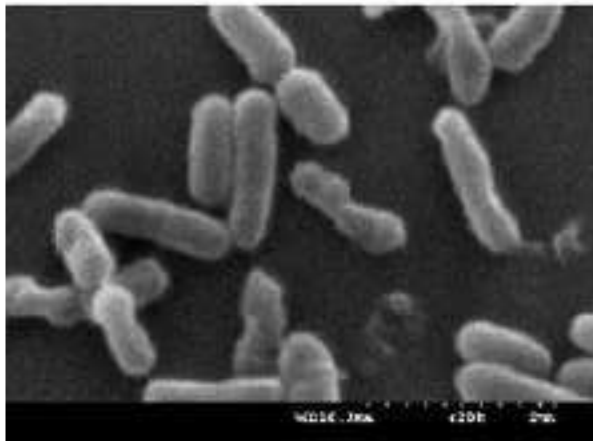
P.aeruginosa | + ***Lithospermi radix*** **(0.1 mg/ml)** | + ***Aloe barbandensis*** **(0.8 mg/ml)**









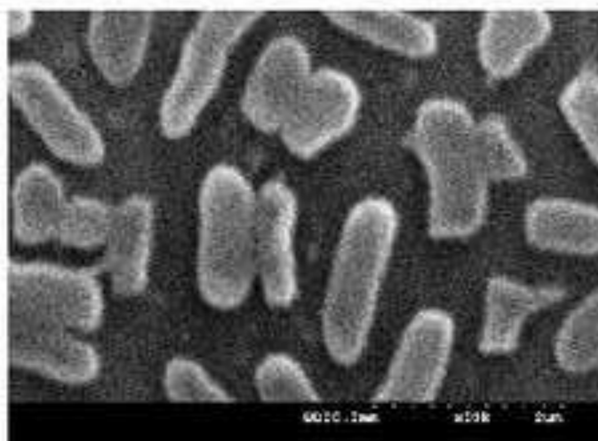


Table 1

sample	**Aloe barbandensis**	**Forsythia suspense**	**Mentha piperta**	**Callicarpa japonica**	**Lithospermi radix**
anti-*Escherichia coli*					
MBC (mg/ml)	12.5	ND	25	50	ND
MIC (mg/ml)	12.5	12.5	25	50	3.2
anti- *Pseudomonas aeruginosa*					
MBC (mg/ml)	6.4	ND	12.5	50	ND
MIC (mg/ml)	25	12.5	12.5	50	3.2
anti- *Staphylococcus aureus*					
MBC (mg/ml)	3.2	ND	6.4	25	ND
MIC (mg/ml)	6.4	6.4	12.5	50	< 0.2

ND: Not Detectable

Table 2

	S. aureus	***E. coli***	***P. aeruginosa***
Lithospermi Radix	0.65 μg/ml	250 μg/ml	0.1 mg/ml
Aloe barbandensis	0.25 mg/ml	0.8 mg/ml	0.8 mg/ml

Table 3

	Spots	Number matches	Percent matches	Increased Spots	Decreased Spots
S. aureus	**355**				
***S. aureus* (6.25 µg/ml *Lithospermi Radix*)**	**328**	**311**	**90.93 %**	**42**	**17**
***S. aureus* (0.25 mg/ml *Aloe barbandensis*)**	**286**	**266**	**82.99 %**	**84**	**20**
E. coli	**301**				
***E. coli* (250 µg/ml *Lithospermi Radix*)**	**289**	**233**	**79.98 %**	**68**	**57**
***E. coli* (0.8 mg/ml *Aloe barbandensis*)**	**275**	**266**	**87.3 %**	**26**	**54**
P. aeruginosa	**289**				
***P. aeruginosa* (0.1 mg/ml *Lithospermi Radix*)**	**163**	**149**	**84.65 %**	**42**	**14**
***P. aeruginosa* (0.8 mg/ml *Aloe barbandensis*)**	**119**	**109**	**70.77 %**	**73**	**10**