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Combined treatment of *Pseudomonas aeruginosa* PA01 biofilm formation with the water-soluble extract of *Ligustrum sinense* and gentamicin sulphate

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Abstract

Ligustrum sinense are commonly used for their anti-inflammatory, anti-rheumatic, diuretic, and hypotensive activities in traditional Chinese medicine. To observe the effects of the combined treatment of a water-soluble extract of *Ligustrum sinense* (WEL) and gentamicin sulphate (GS) on *Pseudomonas aeruginosa* PA01, the micro-dilution method was used to determine the minimal inhibitory concentration (MIC) of GS. Formation of a PA01 biofilm was observed under an optical microscope after treatment with different dosages of WEL and combined treatment with GS. The MIC of WEL was 8g l^{-1}, and permanent activity was also observed. The effect of WEL with GS was synergistic. The motility, biomass of biofilms, and production of pyocyanin of P. aeruginosa were strongly suppressed in the presence of WEL. The conclusion can be drawn that combined antibiotics can be used to treat the contamination due to the biofilm formation caused by *P. aeruginosa*.

Key words

Ligustrum sinense, *Pseudomonas aeruginosa* PA01, Biofilm, Pyocyanin, Synergistic

Introduction

Ligustrum sinense, a member of Oleaceae family has played a very interesting role in the history of medicinal and garden plants. In traditional medicine, the leaves, fruits, and flowers of *L.sinense* have been used for their anti-inflammatory, anti-rheumatic, diuretic and hypotensive activities (Pieroni *et al.,* 2000). Polar fractions and flavones isolated from methanol extracts of the leaves of *Ligustrum* show significant and complementary *in vitro* inhibitory effects on the classical pathway of the complement system (Pieroni *et al.,* 2000). Aqueous extracts of *L. sinense* also show antioxidant activities and chelating effects; (Lau *et al.,* 2002; Wang *et al.,* 2009), exhibit moderate hepato-protective effect (Lau *et al.,* 2002), significantly inhibit the activities of the metallopeptidases angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) at concentration of 100 mgl^{-1}, yielding IC_{50} values of 100 mgl^{-1} for ACE and 50 mgl^{-1} for NEP (Kiss *et al.,* 2008). An *in vitro* inhibitory activity of 58% against the epimastigote form of *Trypanosoma cruzi* has also been observed (Lirussi *et al.,* 2004). *L. sinense* has showed varied kinds of activities and gained more and more interest in expanding its traditional use.

P. aeruginosa is an opportunistic human pathogen that cause diseases in plants, animals, and humans (Lyczak *et al.,* 2000). The potential pathogenicity of *P.aeruginosa* isolates from food animals, retail meat products, and food processing environments was evaluated by determining their antibiotic resistance profiles and invasiveness into human intestinal Caco-2 cell (Kim *et al.,* 2007). The 47 clinical *P.aeruginosa* strains demonstrated the greatest level of resistance to antimicrobials (Hostacka *et al.,* 2006). *P. aeruginosa* has been identified as a principal biofilm-forming opportunistic pathogen in chronic wounds and aquatic systems (Lazaro *et al.,* 2011). Biofilms are clusters of single or mixed cell populations encased in an extracellular matrix that is often found attached to biotic or abiotic substrates

in the environment (Lazaro *et al.,* 2011). Biofilm formation is important because this mode of growth is associated with the chronic nature of the subsequent infections, and with their inherent resistance to antibiotic chemotherapy (Stewart *et al.,* 2011). Periodontitis and chronic lung infection in cystic fibrosis patients are examples of diseases associated with biofilms (Singh *et al.,* 2000). Biofilm-grown bacteria might develop a biofilm-specific biocide-resistant phenotype (Mah *et al.,* 2001). Recent studies (Olson *et al.,* 2002) have demonstrated that selecting antibiotics that are effective for eliminating bacterial biofilms may improve the success rate in treating clinical and experimentally induced disease.

In light of the above, the objective of the present study was to identify an effective approach to cure environmental contamination caused by *P.aeruginosa* and its biofilm formation and reduce the usage of antibiotics commonly used to eliminate a diverse selection of bacterial biofilms.

Materials and Methods

Bacterial strains : The *P.aeruginosa* PA01 strain used in this work was provided by the Institute of Urban Environment, Chinese Academy of Sciences. Luria–Bertani medium containing various concentrations of water soluble extracts of *L.sinense* (WEL) were used for bacterial growth under rapid shaking at 37°C.

Plant material : The plant material was collected from the campus of Fujian Agriculture and Forestry University campus. Leaves were dried, grounded into powder, and boiled in distilled water for 30 min. The solvent was then evaporated under reduced pressure. The extract was flushed in a D_{101} column and eluted with distilled water and methanol. The methanolic extract was concentrated under vacuum and then stored until use.

Antibiotic resistance of *P. aeruginosa* PA01 with WEL or GS : The standard antibiotic disc diffusion method was used according to the method of Szpakowska with some modification (Szpakowska *et al.,*1997). Briefly, dilutions of WEL at multiple proportions were prepared at final concentrations of 12, 11, 10, 9, 8, 7, 6, 5, 4, and 3 gl^{-1}, and a 96-well plate was used for each drug, blank and positive controls were set accordingly. Each sample was prepared at various concentrations and added to the wells at a volume of 10 µl. The original density of bacteria was 1.5×10^8 CFU ml^{-1}, and the working concentration was diluted with Muller-Hinton broth at a dilution of 1:1000, after which 100 µl of the resulting solution was added to each well. The plate was incubated at 37°C for 24 and 48 hr. The lowest concentration of drugs without bacteria in wells treated for 24 hr was defined as the minimal inhibitory concentration (MIC), the lowest concentration of drugs without bacteria in wells treated for 48 hr was defined as the minimal bactericidal concentration (MBC). Gentamicin (GS) experiments were formed in the same manner.

Combined effects of antibiotics and WEL : The chessboard microdilution method was used to evaluate the effects of antibiotics and WEL according to the method of Jayaraman with some modification (Jayaraman *et al.,* 2010). Briefly, two fold serial dilutions of the antibiotic and two fold serial dilutions of the samples were prepared for every combination tested and 90 µl of different concentrations of WEL and 10 µl of different concentrations of GS were placed into the wells of the sterile 96-well microtiter plate. The inoculums were prepared using the above described MIC determination method, and the microtiter plates were then incubated at 35 °C and MIC was determined after 24 hr of incubation. The fractional inhibitory concentration (FIC index) for all the combinations was determined using the following formula:

$$\text{FIC index} = FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$$

(FIC_A, FIC_B - Fractional inhibitory concentration of drug A and B respectively; MIC_A, MIC_B - Minimum inhibitory concentration of drug A and B respectively; [A], [B] - Concentration of drug A and B respectively). FIC index by checkerboard method is interpreted as follows: FIC$\leq$ 0.5 - indicates synergy effect; FIC> 0.5 and $\leq$ 4 - indicates additive effect; and FIC> 4 – indicates antagonism effect).

Motility assays : The motility of PA01 was assayed according to the method of Rashid *et al.* (2000) with some modification. Swimming media used for assay was tryptone broth (10 gl^{-1} tryptone, 5 gl^{-1} NaCl) that contained 0.3% (w/v) agarose. Swim plates were inoculated with bacteria from an overnight culture in LB agar (1.5%, w/v) plates at 37 °C with a sterile toothpick. The plates were then wrapped with Saran Wrap to prevent dehydration and incubated at 30 °C for 12–14 hr. Swarming media used for assay consisted of 0.5% (w/v) Difco bacto-agar with 8 gl^{-1} Difco nutrient broth, to which 5 gl^{-1} glucose was added. Swarm plates were typically allowed to dry at room temperature overnight before being used. Swarming efficiency was improved when cells were inoculated onto swarm plates from swim agar plates incubated overnight at 30 °C; inoculation from overnight LB agar plates also supported swarming.

Establishment of *in vitro* biofilm model : The biofilm formation assay used is based on the method of Coenye *et al.* (2010) with some modification (Extremina *et al.,* 2011; Lin *et al.,* 2006; Pitts *et al.,* 2003; Rinaudi *et al.,* 2006). This assay relies on the ability of the cells to adhere to the wells of 96-well microtiter dishes made of polyvinylchloride. The PA01 suspension (1 ml) was inoculated in 100 ml of tryptic soy broth (TSB), then, 50 µl from an overnight culture, coupled with different concentrations of WEL (diluted with TSB

medium), was added into the wells of a 96-well plate and cultured at 37 °C for 24 hr. After inoculation, the plates were covered with plastic to prevent evaporation and incubated without agitation at 37 °C. The wells were washed three times with 180 µl of sterile physiological saline solution, and the plates were vigorously shaken in each wash in order to remove all non-adherent bacteria. The plates were emptied, left to dry, and stained for 30 min with 125 µl per well of 0.1% crystal violet (CV), then rinsed thoroughly and repeatedly with water and scored for biofilm formation, the plate was placed in a 50 °C drying oven, The CV-stained biofilm was solubilized in 200 µl of glacial acetic acid, a 20 µl portion of the solution was placed into a new 96-well plate, in which 180 µl of 95% ethanol had been placed in advance. The absorbance at OD_{590} was detected by a microplate reader. The negative control was set as a mixture without the sample solution, and the blank control was set as a mixture without bacteria inoculation. The CV-stained biofilm was solubilised in 150 µl of 95 % ethanol and the absorbance determined at 595 nm in a spectrophotometer.

Detection of pyocyanin production : The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution (Essar *et al.,* 1990; Krom *et al.,* 2007). Bacteria were grown in PB medium (Bacto-peptone 20 gl^{-1}, $MgCl_2$ 1.4 gl^{-1}, K_2SO_4 10 g l^{-1}, pH 7.0). A 1 ml culture of PA01 was combined with 50 ml of different concentrations of WEL in a 250 ml flask, cultured at 37 °C, and shaken at 120 rpm for 12 hr. Five microliters of cell-free culture supernatant was extracted with 3 ml of chloroform for 2 hr. Chloroform was decanted and subsequently extracted with 1 ml of 0.2 M HCl to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per millilitre of culture supernatant were determined by multiplying the optical density at 520 nm by 17.072.

Results and Discussion

Antibiotic resistance of *P. aeruginosa* PA01 with WEL or GS : The MICs of WEL for antibiotics against *P.aeruginosa* are shown in Table 1. PA01 did not grow when the WEL concentration was above 8×10^3 mgl^{-1} and culturing was carried out for 24 hr. In contrast, normal growth was observed at WEL concentrations below 8×10^3 mgl^{-1} and cultured for 24 hr. Hence, 8×10^3 mgl^{-1} was chosen as the MIC of WEL. PA01 did not grow at concentrations of WEL above 9×10^3 mgl^{-1} and cultured for 48 hr. Therefore, 9×10^3 mg l^{-1} was chosen as the MBC of WEL.

From Table 2, it can be seen that the MIC and MBC of GS were lowest among the antibiotics surveyed, indicating that it is the best antibiotic against *P. aeruginosa* PA01. The strain was significantly inhibited by GS, even after 24, 36 and 48 hr of culture. GS retained its ability to inhibit PA01 growth. Thus, GS was chosen for further studies on the combined effects of drugs.

Combined effects of antibiotics and WEL : The results of the combined effect of WEL and GS are shown in Table 3. FIC_A (WEL) and FIC_B (GS) was 1.35/8 and 2/80 respectively. The FIC index was 0.194, which is less than 0.5, signifying a synergistic effect. Application of combined WEL and GS significantly reduced the MIC of WEL.

Motility assays : Under the same culture conditions with different concentrations of WEL, the swimming motility of PA01 was limited. The effect on PA01 movement with different concentration of WEL is shown in Fig. 1 and Fig. 2. The diameter of the blank was 3.91 cm. At WEL concentrations of 6, 4, and 2 ($\times10^3 mgl^{-1}$), the diameters decreased to 2.89, 1.87 and 1.25 cm respectively, indicating limited movement Surface translocation on the surface of agar 0.45% or more in concentration supports a swarming motility among some bacteria that is clearly distinguishable from swimming. We observe that *P. aeruginosa* also possess such a swarming motility (Fig. 3a). Irregular branching began to appear at the periphery when the colony grow to 2–4 mm in diameter in 12–24 hr depending on the medium and agar. Fig. 3 and Fig. 4 shows the delayed cluster movement of PA01. In untreated samples, the strain can cover the entire plate within 1 to 2 d. Addition of WEL (6×10^3 mgl^{-1}) prolonged the coverage time to 4 d. The swarming motility of PA01 was inhibited by treatment with WEL, indicating that the extract can prevent the infection and migration of PA01 by inhibition of flagella movement. The biofilm production of PA01 *in vitro* was assessed using its OD_{590} and compared with the growth control. Fig. 5 shows that the biomass of the film formed was identical to that of the blank control when the concentration

Table 1 : MICs of WEL against PA01

Time (hr)	WEL concentration ($\times10^3$ mgl^{-1})											
	3	4	5	6	7	8	9	10	11	12	CK1	CK2
12	+	+	-	-	-	-	-	-	-	-	+	-
24	+	+	+	+	+	-	-	-	-	-	+	-
36	+	+	+	+	+	+	-	-	-	-	+	-
48	+	+	+	+	+	+	-	-	-	-	+	-

+: the growth of bacteria; -: sterile growth; CK1: growth control; CK2: blank control

Table 2 : MICs of GS against PA01

Antibiotics	MIC ($\times10^3\mu gl^{-1}$)	MBC ($\times10^3\mu gl^{-1}$)
GS sulphate	80	80
Ampicillin sodium	320	640
Ampicillin sodium	80	160
Streptomycin sulphate&	312.5	625
Anamycin	320	320
Etracycline	640	1280

The concentrations of streptomycin sulphate are 10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.0625, and 19.5 ($\times10^3$) Ul^{-1}

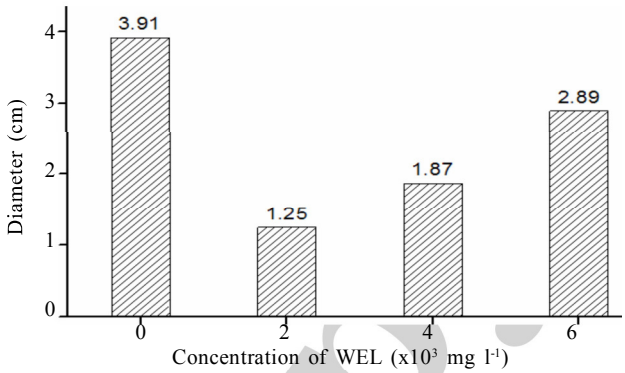

Fig. 1 : The effect of combined treatment on swimming motility.

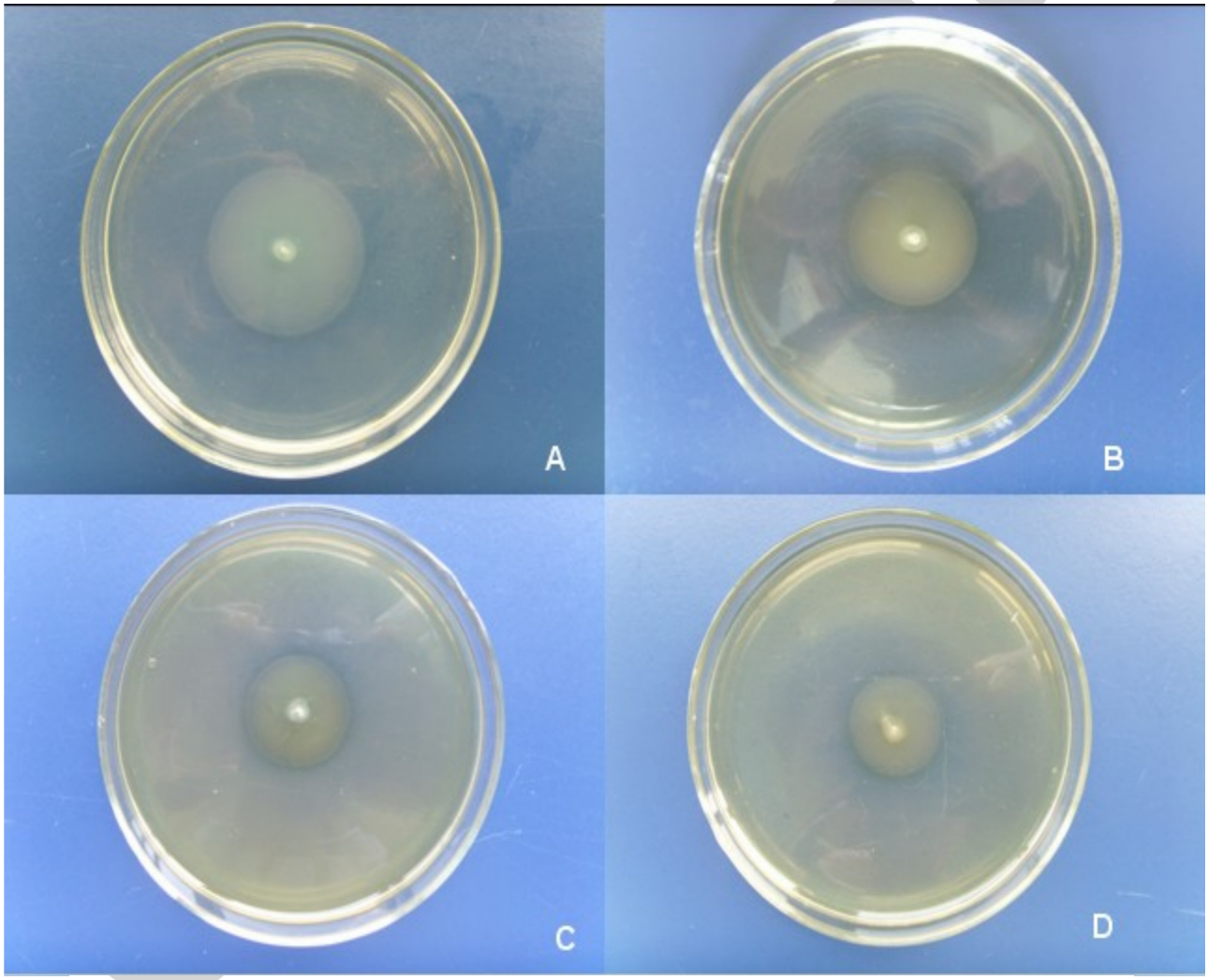

Fig. 2 : The effect of WEL against swimming motility ($\times10^3 mgl^{-1}$). (A) Blank control; (B) 6; (C) 4 ;(D) 2

of WEL was below 4×10^3 mgl^{-1}. However, the film biomass was significantly decreased when the concentration of WEL was 4×10^3 mgl^{-1} and above.

Detection of pyocyanin production : The effect of WEL treatment on pyocyanin production was evaluated, and the results are shown in Fig. 6 and 7. From Fig. 6, it can be seen that the blank control produced 2.82 gl^{-1} pyocyanin, when PA01 was treated with WEL at concentrations of 4 gl^{-1} or above, the pyocyanin yield decreased by at least 59.2%.

Table 3 : *In vitro* activity of combined treatment against PA01

GS ($\times10^3$ µgl^{-1})	WEL ($\times10^3$ mgl^{-1})											
	5.4	4.95	4.5	4.05	3.6	3.15	2.7	2.25	1.8	1.35	CK1	CK2
16	-	-	-	-	-	-	-	-	-	-	+	-
8	-	-	-	-	-	-	-	-	-	-	+	-
4	-	-	-	-	-	-	-	-	-	-	+	-
2	-	-	-	-	-	-	-	-	-	-	+	-
1	-	-	-	+	+	+	+	+	+	+	+	-
0.5	-	-	-	+	+	+	+	+	+	+	+	-
0.25	-	-	-	+	+	+	+	+	+	+	+	-
0.125	-	-	-	+	+	+	+	+	+	+	+	-

+: growth of bacteria; - : sterile growth; CK1: growth control; and CK2: blank control. Each test was replicated thrice.

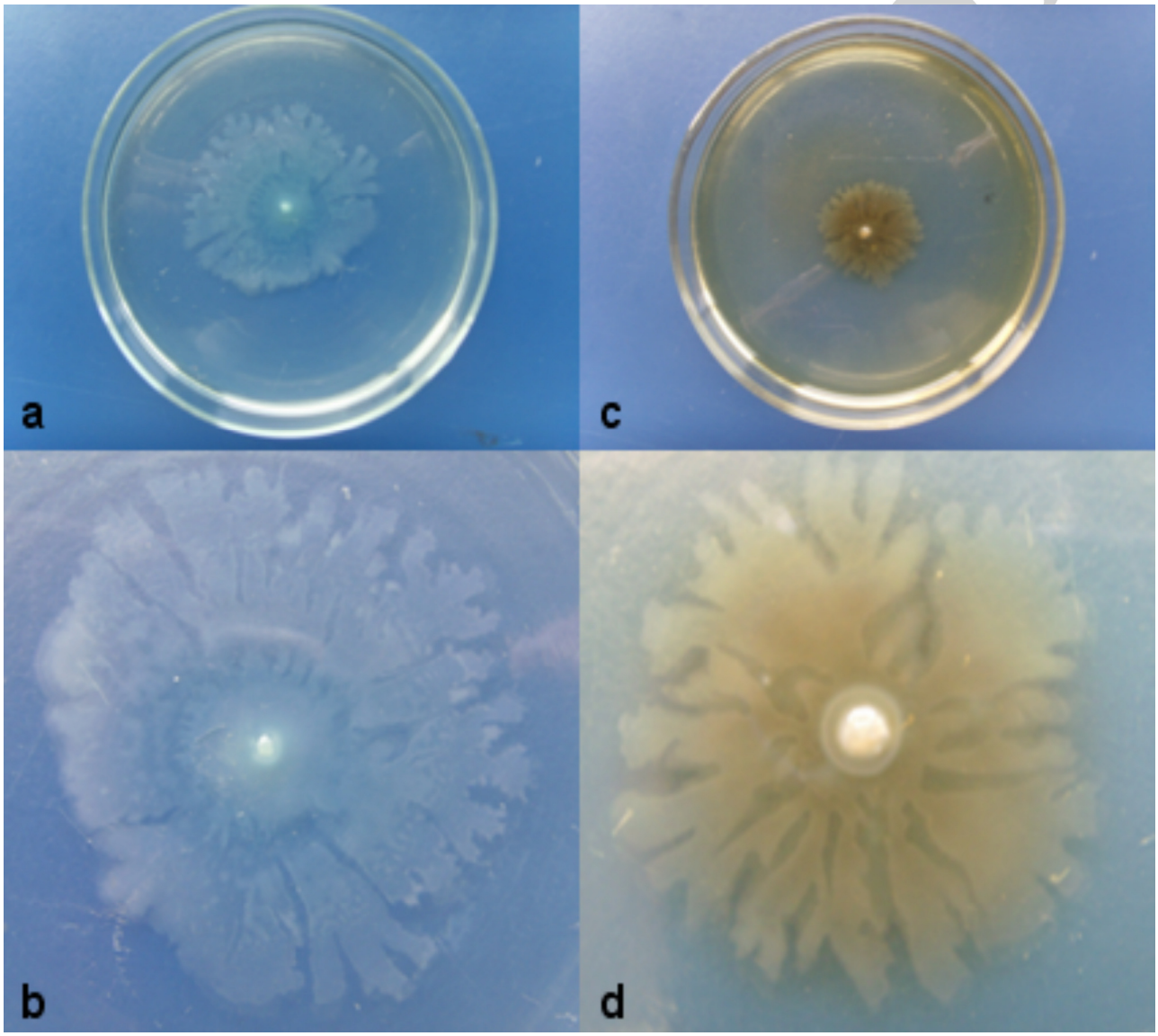

Fig. 3 : Inhibitory effects of WEL (6.0×10^3mgl^{-1}) on swarming motility: (a) Blank control; (b) enlarged photo of the blank control; (c) sample-treated control; (d) enlarged photo of the sample-treated control

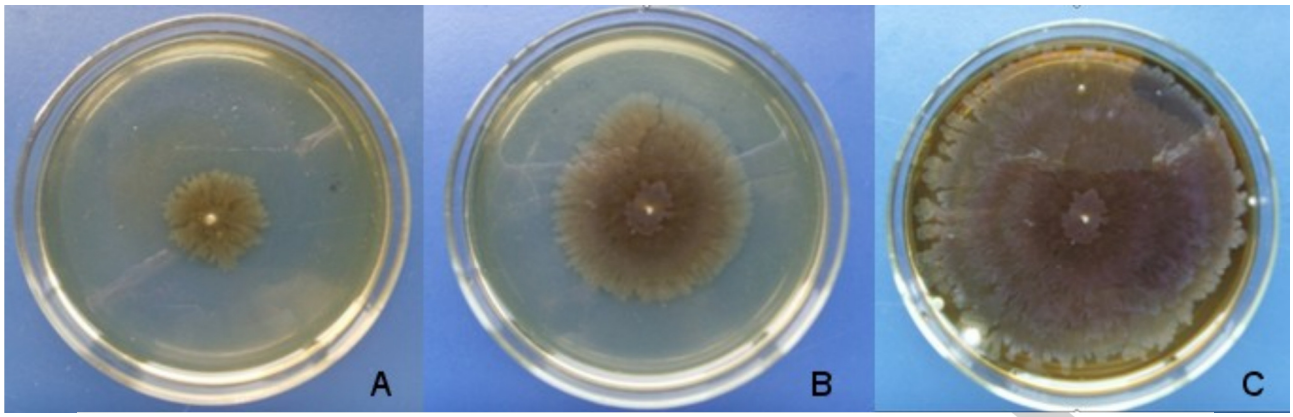

Fig. 4 : Changes in swarming motility**:** (A) Day 1; (B) Day 2 ;(C) Day 3

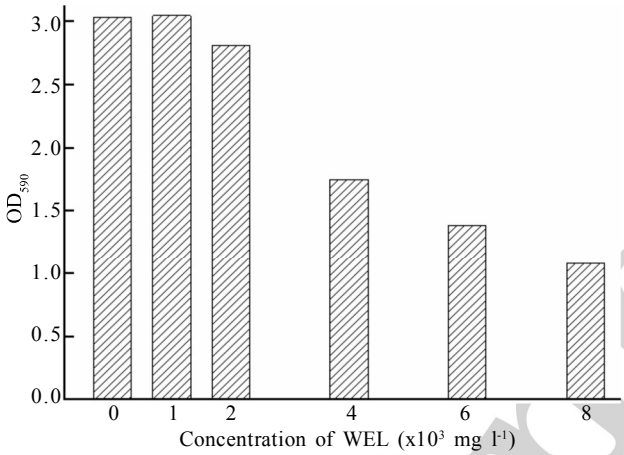

Fig. 5 : Effects of WEL on the PA01 film biomass

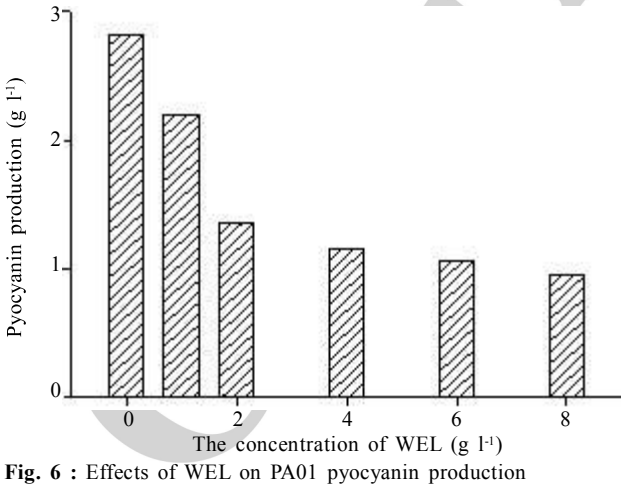

Fig. 6 : Effects of WEL on PA01 pyocyanin production

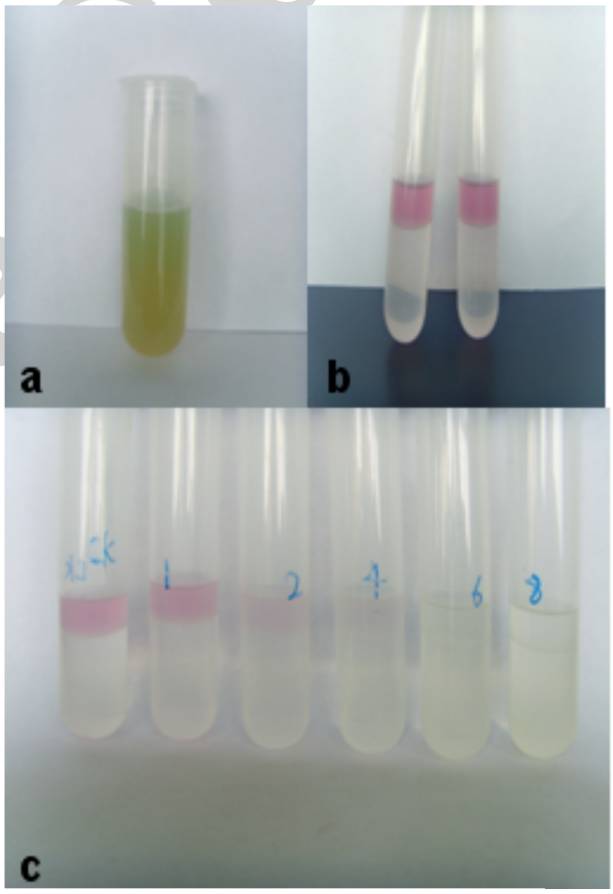

Fig 7 : Effects of WEL on PA01 pyocyanin production: (a) Non-treated sample; (b) treated sample; (c) pyocyanin production of PA01 treated with different concentrations of WEL

Pyocyanin production was significantly inhibited by WEL. The change in colour is evident in Fig. 7, proving the inhibitory effect of the extract indirectly. The current study showed that the biofilm formation of *P.aeruginosa* PA01 was inhibited by WEL. Its inhibition was reflected in three respects: the limited range of motility, the reduction in film biomass and decrease in pyocyanin production. This study clearly demonstrates that 1.35×10^3 mgl^{-1} WEL with 2 mgl^{-1} GS has the ability to inhibit the growth of PA01, which MIC is 8×10^3 mgl^{-1} and 80 mgl^{-1} respectively.

The effect of different drugs on *P.aeruginosa* has been extensively studied, such as the effect of liposomal amikacin on *P. aeruginosa* (Antos *et al.,* 1995). Liposomal amikacin proved to be active as a bactericidal agent after 3 hr of incubation with *P.aeruginosa.* Several studies have focused on the virulence factor of alkaline protease, proving that flagellin plays a major role in *P.aeruginosa* virulence (Bardoel *et al.,*, 2012). However, to the best of our knowledge, the effect of WEL on *P.aeruginosa* and its biofilm formation is meagre.

Since around 800 B.C., the genus *Ligustrum* has been used in traditional Chinese medicine as a valued tonic that was often mixed into soup and wine, due to its liver-protective immunomodulatory and anti-mutagenic effects, plants of this genus have been suggested for the prevention/ therapy of many diseases, mainly in Oriental areas (Adriana *et al.,*, 2008). In recent years, efforts have been made to validate its use. This study suggests that *Ligustrum* is a potentially promising remedy to treat *P. aeruginosa* contamination in future.

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References

Hostacka, A., I. Ciznar, L. Slobodnikova and D. Kotulova: Clinical *Pseudomonas aeruginosa:* Potential factors of pathogenicity and resistance to antimicrobials. *Folia Microbiol (Praha)*, **51**, 633-638 (2006).

Pieroni, A. and P. Pachaly: An ethnopharmacological study on common privet (*Ligustrum vulgare*) and phillyrea (*Phillyrea latifolia*). *Fitoterapia*, **71**, 89-94 (2000).

Adriana, A., M. Pavol, S. Pavel, N. Milan, F. Viera and K. Magdalena: Effects of *Ligustrum delavayanum* Hariot on cholesterol levels and arrhythmogenesis induced by myocardial ischaemia-reperfusion injury in diabetic-hypercholesterolaemic rats. *Environ. Toxicol. Pharmacol.,* **26**, 255-258 (2008).

Antos, M., E.A. Trafny and J. Grzybowski: Antibacterial activity of liposomal amikacin against *Pseudomonas aeruginosa in vitro*. *Pharmacolo. Res.*, **32**, 85-87 (1995).

Bardoel, B.W., K.P.Mv. Kessel, J.Gv. Strijp and F.J. Milder: Inhibition of *Pseudomonas aeruginosa* virulence: Characterization of the AprA-AprI interface and species selectivity. *J. Mol. Biol.,* **415**, 573-583 (2012).

Coenye, T. and H.J. Nelis: *In vitro* and *in vivo* model systems to study microbial biofilm formation. *J. Microbiol. Meth.,* **83**, 89-105 (2010).

Essar, D.W., L. Eberly, A. Hadero and I.P. Crawford: Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: Interchange ability of the two anthranilate synthases and volutionary implications. *J. Bacteriol.,* **172**, 884-900 (1990).

Extremina, C.I., L. Costa, A.I. Aguiar, L. Peixe and A.P. Fonseca: Optimization of processing conditions for the quantification of enterococci biofilms using microtitre-plates. *J. Microbiol. Meth.*, **84**, 167-173 (2011).

Jayaraman, P., M.K. Sakharkar, C.S. Lim, T.H. Tang and K.R. Sakharkar: Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa in vitro*. *Int. J. Biol. Sci.,* **6**, 556-568 (2010).

Singh, K.P., L.A. Schaefer, R.M. Parsek, O.T. Moningerk, J.M. Welsh and E.P. Greenberg: Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature,* **407**, 762-764 (2000).

Kim, S.H. and C.I. Wei: Antibiotic resistance and Caco-2 cell invasion of *Pseudomonas aeruginosa* isolates from farm environments and retail products. *Int. J. Food Microbiol.,* **115**, 356-363 (2007)

Kiss, A.K., M. Mank and M.F. Melzig: Dual inhibition of metallopeptidases ACE and NEP by extracts, and iridoids from *Ligustrum vulgare* L. *J. Ethnopharmacol*, **120**, 220-225 (2008)

Krom, B.P., J.B. Cohen, G.E. McElhaney Feser and R.L. Cihlar: Optimized candidal biofilm microtiter assay. *J. Microbiol. Meth.,* **68**, 421-423 (2007).

Lau, K.M., Z.D. He, D. Dong, K.P. Fung and P.P.H. But: Anti-oxidative, anti-inflammatory and hepato-protective effects of *Ligustrum robustum*. *J. Ethnopharmacol,* **83**, 63-71 (2002)

Lin, C.T., Y.J. Huang, P.H. Chu, J.L. Hsu, C.H. Huang and H.L. Peng: Identification of an HptB-mediated multi-step phosphorelay in *Pseudomonas aeruginosa* PA01. *Res. Microbiol.,* **157**, 169-175 (2006).

Lirussi, D., J. Li, J.M. Prieto, M. Gennari, H. Buschiazzo, J.L. Rios and A. Zaidenberg: Inhibition of *Trypanosoma cruzi* by plant extracts used in Chinese medicine. *Fitoterapia,* **75**, 718-723 (2004)

Lyczak, J.B., C.L. Cannon and Piera: Establishment of *Pseudomonas aeruginosa* infection:lessons from a versatile opportunist. *Microbes. Infect.,* **2**, 1051-1060 (2000)

Mah, T.F.C. and G.A.O. Toole: Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.,* **9**, 34-39 (2001)

Olson, M.E., H. Ceri, D.W. Morck, A.G. Buret and R.R. Read: Biofilm bacteria: Formation and comparative susceptibility to antibiotics. *Can. J. Vet. Res.,* **66**, 86-92 (2002)

Pieroni, A., P. Pachaly, Y. Huang, B.V. Poel and A.J. Vlietinck: Studies on anti-complementary activity of extracts and isolated flavones from *Ligustrum vulgare* and *Phillyrea latifolia* leaves (Oleaceae). *J. Ethnopharmacol.,* **70**, 213-217 (2000).

Pitts, B., M.A. Hamilton, N. Zelver and P.S. Stewart: A microtiter-plate screening method for biofilm disinfection and removal. *Microbiol. Meth.,* **54**, 269-276 (2003)

Rashid, M.H. and A. Kornberg: Inorganic polyphosphate is needed for swimming and twitching motilities of *Pseudomonas aeruginosa*. *PNAS,* **97**, 4885-4890 (2000).

Rinaudi, L., N.A. Fujishige, A.M. Hirsch, E. Banchio, A. Zorreguieta and W. Giordano: Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation. *Res. Microbiol.,* **157**, 867-875 (2006)

Sanz-Lazaro, C., F. Navarrete-Mier and A. Marin: Biofilm responses to marine fish farm wastes. *Environ. Pollut.,* **159**, 825-832 (2011).

Stewart, P.S. and J.W. Costerton: Antibiotic resistance of bacteria in biofilms. *Lancet.,* **358**, 135-138 (2001).

Szpakowska, M., J. Reiss, A. Graczykb, S. Szmigielski, K. Lasocki and J. Grzybowski: Susceptibility of *Pseudomonas aeruginosa* to a photodynamic effect of the arginine hematoporphyrin derivative. *Int. J. Antimicrob. Ag.,* 8, 23-27 (1997)

Wang, Z.H., C.C. Hsu and M.C. Yin: Antioxidative characteristics of aqueous and ethanol extracts of glossy privet fruit. *Food Chemistry*, **112**, 914-918 (2009).