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Herbal Sensitivity of *Pseudomonas* Bacteria Isolated from Cultured Tilapia with Useful Applications in Vaccine Preparation

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ABSTRACT

The antibacterial activity of certain commercial antibiotics and common herbs was evaluated against pathogenic *Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas aeruginosa* isolated from Malaysian and Egyptian cultured fish, mainly tilapia. A suspension of freshly cultured isolates was prepared (with 0.5 OD) and 100 μL of this suspension was spread over the Muller's Hinton agar plates. The antibiotic discs were inoculated on each cultured plate while the herbal extracts were soaked on Whatman filter paper (20 μL each) that have been cut into discs and later inserted on to bacteria-cultured plates to screen their sensitivity to both antibiotics and herbs. Double-fold dilution was used to determine the Minimal Inhibitory Concentration (MIC) for the effective herbs at 100, 50, 25, 12.5 and 6.25%. Results revealed high resistance of the tested bacteria against most of the screened antibiotics except Ciprofloxacin. With regard to herbal sensitivity, only *Origanum vulgare* showed effectiveness and inhibition zone against all isolates. The MIC ranged from 15-40% for both Egyptian and Malaysian isolates. Thus, *Origanum vulgare* is recommended as a feed additive for cultured fish and can also be applied for inactivated and live-attenuated *Pseudomonas* vaccines' preparation.

Key words: *Pseudomonas fluorescens, P. putida, P. aeruginosa,* herbs, *Origanum vulgare*

INTRODUCTION

Pseudomonas is a genus of gamma *proteobacteria* and it belongs to the larger family of *pseudomonads*. *Pseudomonas* have aerobic metabolism and are able to grow on a wide variety of organic substrates. Most *pseudomonads* are free-living saprophytic organisms in soil or water where they play an important role in decomposition, biodegradation and in the carbon and nitrogen cycles. Consequently, they are important organisms in bioremediation (Gyles *et al*., 2010).

Pseudomonas putida is a Gram-negative rod-shaped saprotrophic soil bacterium. It is the first patented organism in the world because it demonstrates a very diverse metabolism, including the ability to degrade organic solvents such as toluene. Because of this ability, *Pseudomonas putida* has been put to use in bioremediation and to biodegrade oil. It is preferable to some other *Pseudomonas* species capable of such degradation as it is a safe strain of bacteria compared to

P. aeruginosa which is an opportunistic human pathogen rather than beneficial (Ward *et al.*, 2006). In aquaculture, *Pseudomonas* spp. (*P. fluorescens, P. putida* and *P. aeruginosa*) were incorporated in severe outbreaks among *O. niloticus* in fish hatcheries (Ahmed and Shoreit, 2001), intensive culture farms (Eisa *et al.*, 1993), earthen ponds and floating cages (Abu El-Attah, 2003; Abdel-Hadi *et al.*, 2008). Resistance of *Pseudomonas* spp. against the most commonly used antimicrobials in aquaculture has developed greatly in the recent years (Inglis *et al.*, 1997; Ahmed and Shoreit, 2001; Taylor, 2003). Thus, this study was conducted to study the antibiotic sensitivity of *Pseudomonas* bacteria isolated from Egypt and Malaysia, herbal sensitivity of these bacteria to some common herbs used in aquaculture and to determine the Minimal Inhibitory Concentrations (MIC) of the effective herbs.

MATERIALS AND METHODS

Bacterial isolates: Six bacterial isolates belonging to three *Pseudomonas* species including *P. fluorescens, P. putida* and *P. aeruginosa,* were used in this study. They were isolated from cultured tilapia (Abdel-Hadi, 2004) in Egypt (3 isolates) and Malaysia (3 isolates). These bacteria were used to determine their sensitivity towards certain antibiotics as well as herbal extracts and oils, commonly-used in aquaculture.

Culture media: A suspension of freshly cultured isolates was prepared in Tryptic Soy Broth (TSB) (with 0.5 OD) and 100 μL of this suspension was spread over Muller's Hinton agar plates. The antibiotic discs were inoculated on each cultured plate while the herbal extracts were soaked on Whatman filter paper (20 μL each) that have been cut into discs and then inserted on to bacteria-cultured plates to screen their sensitivity to the screened herbs.

Antibiotic discs: Ciprofloxacine, gentamycin, cephaloxin, chloramphenicol, ampicillin/sulbactam, sulphamethoxazole/trimethoprim, erythromycin, novobiocine, vancomycin, nitrofurantoin (Becton, Dickinson and company, DB, USA).

Herbal extracts and oils: Prepared using crude methanolic extraction and oils were ready made. *Origanum vulgare* (commonly known as wild marjoram), *Matricaria chamomella* (German chamomile), *Allium sativum* (garlic), *Zingiber officinale* (ginger), *Origanum majorana* (marjoram) and *Artemesia cina* (wormseed plants) extracts as well as *Nigella sativa* (black seed), *Cinnamomum camphora* (camphor) and *Azadirachta indica* (Neem) oils. Methanolic extraction was done according to Sahin *et al.* (2004).

Antibiogram assay: Was conducted using Muller's Hinton agar, infusion of bacteria, sticking the Antibiotic discs and incubating for 18 h before reading the results and measuring the inhibition zones (Muniruzzaman and Chowdhury, 2004).

Herbal sensitivity: Using well diffusion agar assay and filter paper discs soaked with 20 μL of herbal oils and extracts (Hakan *et al.*, 2009).

Minimal inhibitory concentration (MIC): Conducted only towards the effective herbs to detect the MIC of the double-fold diluted herbs against the 6 bacterial isolate. Double-fold dilution was used to determine the Minimal Inhibitory Concentration (MIC) for the effective herbs at 100, 50, 25, 12.5 and 6.25%. One hundred microliter of these dilutions for each isolates were inoculated

on TSA plates and were incubated for 24 h at 25°C (Palavesam *et al*., 2006). Further investigation was conducted to detect the precise value of MIC. This was done via using less dilutions than the preliminary MIC, which were determined by the double-fold dilution method. *O. vulgare* concentration of 15, 18, 21 and 24% were used for a preliminary MIC of 25% as well as 30, 35, 40 and 45% were tested when preliminary MIC was 50%.

Vaccine preparation: Free cells of the Egyptian *Pseuomonas putida* vaccine was chosen because of the high Pathogenicity and prevalence of this bacterial species isolated from a previous study (Abdel-Hadi, 2004).

Inactivated *P. putida* vaccine was inactivated using concentrations equal or higher than the MIC (25, 50 and 100% of *O. vulgare* extract). The free cells were cultured, adjusted to a concentration of 10^{10} then harvested and washed by PBS (Abdel-Hadi *et al*., 2009). However, the heating step was skipped and replaced by adding 10 mL of the herbal extract to the bacterial pellet-containing test tubes and suspending for 1, 12 and 24 h. Sterility test was conducted by culturing the herb-bacterial suspension onto TSA plates and incubation for 24 h.

Live-attenuated *P. putida* vaccine was prepared using 25% *O. vulgare* (less than the MIC which is 40%) according to Abdel-Hadi *et al*. (2009).

RESULTS

Antibiotic sensitivity: Results of this study revealed that the 6 tested *Pseudomonas* species were resistant to most of the screened antibiotics except Ciprofloxacin, which inhibited the growth of all Egyptian and Malaysian isolates, followed by Gentamycin, which inhibited all Malaysian isolates and 1 Egyptian isolate only (*P. aeruginosa*) but didn't inhibit the growth of the Egyptian *P. fluorescens* and *P. putida* at all (zero inhibition zone). On the other hand, the Malaysian *P. putida* was sensitive to all tested antibiotics except ampicillin/sulbactam, erythromycin and nitrofurantoin. Chloramphenicol, novobiocin and vancomycin couldn't inhibit the growth of all tested *Pseudomonas* bacteria except this Malaysian isolate of *P. putida* (Table 1).

Table 1: Commercial antibiotics sensitivity test against bacterial isolates, measured by the diameter of inhibition zones

	Zone diameter interceptive standard of antibiotics			*P. fluorescens* (mm)		*P. putida* (mm)		*P. aeruginosa* (mm)	
Antibiotic	R	I	S	E	M	E	M	E	M
C30: Chloramphenicol	≤12	13-17	≥18	8.9	0	10.3	52.5	14.5	0
SAM20: Amplicillin/Sulbactam	≤11	12-14	≥15	12.5	0	0	0	12.5	0
CL30: Cephaloxin	≤14	15-17	≥18	0	0	0	20.5	0	0
CIP5: Ciprofloxacin	≤15	16-20	≥21	28.9	52.5	24.5	39.5	26.5	41.5
SXT25: Sulphamethoxazole/ Trimethoprim	≤10	11-15	≥16	0	0	0	52.5	0	0
E15: Erythromycin	≤13	14-22	≥23	0	20.5	0	0	0	2.45
CN10: Gentamicin	≤12	13-14	≥15	0	39.5	0	20.5	21.5	51.5
NV5: Novobiocine	≤17	18-21	≥22	0	0	0	39.5	0	0
VA30: Vancomycin	≤14	15-16	≥17	0	0	0	52.5	0	0
F50: Nitrofurantoin	≤14	15-16	≥17	2.45	0	0	0	8.9	0

R: Resistant, I: Intermediate, S: Sensitive, E: Egyptian, M: Malaysian, R: Resistant

Table 2: Minimal inhibitory concentrations (MIC) and inhibition zones, indnced by *O. vulgare* extract against bacterial isolates from cultured tilapia fish in the Egypt and Malaysia

	Bacterial isolates		
O. vulgare	*P. fluorescens*	*P. putida*	*P. aeruginosa*
Egyptian isolates			
Inhibition zone (mm)	13	12.5	13.0
MIC (%)	15	40.0	35.0
Malaysian isolates			
Inhibition zone (mm)	12	12.5	13.6
MIC (%)	30	25 .0	25.0

Herbal sensitivity: Six tested *Pseudomonas* isolates were resistant to all screened herbal extracts and oils except the methanolic extract of wild marjoram; *Origanum vulgare,* which showed a strong antibacterial activity and inhibition zone against all isolates. The MIC range was 15-40% for the Egyptian isolates and 25-30% for the Malaysian isolates. There was no significant difference between the Egyptian and the Malaysian bacterial isolates regarding the size of inhibition zone, which ranged between 12 and 13.6 mm for both. However, estimates of the Minimal Inhibitory Concentration (MIC) were greatly lower for the Malaysian *P. putida* and *P. aeruginosa* (25% for both) than their Egyptian counterparts (40 and 35%, respectively). On the contrary, only the Malaysian *P. fluorescens* had a higher MIC figure (30%) than the Egyptian counterpart (15%) (Table 2).

Vaccine preparation

Inactivated *P. putida* vaccine: Sterility test showed that there was no bacterial growth indicated by no colony development was noticed after 1, 12 or 24 h for the 40% (MIC), 50 and 100% of *O. vulgare*-free bacterial cell suspension. However, there was bacterial growth and colony for the bacterial suspension in 25% of the wild Marjoram herbal extract after 1 and 12 h but not after 24 h.

Live-attenuated *P. putida* vaccine: Very small pin point-sized weak colonies grew on TSA plates indicating the attenuation of the bacterial vaccine in just 1 simple step.

DISCUSSION

Antibiotic sensitivity: From results of Table 1 and 2, it could be indicated that the Egyptian *Pseudomonas* isolates showed relatively, more tolerance than the Malaysian counterparts towards both antibiotics and herbs. This may be attributed to the climatic contrast between both countries, where the Egyptian climate is hot and arid, while the Malaysian tropical climate is characterized by hot, humid and rainy forests, which are very rich in organic matter and other requirements for bacterial growth. The hot and dry climate in Egypt as a subtropical country could be the reason of the relative high tolerance of the Egyptian isolates. Most *Pseudomonads* are free-living saprophytic organisms in soil and water (Gyles *et al*., 2010). *Pseudomonas* tolerance to antibiotics was also recorded by Ahmed and Shoreit (2001) and Taylor (2003).

Herbal sensitivity: Only methanolic extract of *Origanum vulgare,* showed an antibacterial activity and inhibition zone against all isolates. The antiurolithic effect was confirmed by

Khan *et al.* (2011). This could be attributed to *O. vulgare* chemical constituents, which include carvacrol, thymol, limonene, pinene, ocimene and caryophyllene. The leaves and flowering stems are strongly antiseptic, antispasmodic, carminative, cholagogue, diaphoretic, emmenagogue, expectorant, stimulant, stomachic and mildly tonic (Peter, 2004).

Vaccine preparation

Inactivated *P. putida* vaccine: The 40-50% of *O. vulgare* could be used for inactivated *P. putida* vaccine preparation instead of heat or formalin inactivation. This environmentally-friendly herbal adjuvant could have the potential to replace the chemical adjuvant such as formalin and aluminium salts, which have serious adverse effects. Herbal immune-modulators are paving their way as safe alternatives (Sakure *et al.*, 2008).

Live-attenuated *P. putida* vaccine: Very small pin point-sized weak colonies grew on TSA plates indicating the attenuation of the bacterial vaccine in just 1 simple step. This is much easier than the commonly used serial passage method in an unusual host or animal rather than fish, which is expensive, laborious and time consuming. These results were similar to those recorded by Abdel-Hadi *et al.* (2009).

It could be concluded that methanolic extract of *Origanum vulgare* has the potential to be used as a new herbal adjuvant for both inactivated (50%) and live-attenuated (25%) *Pseudomonas* vaccines against the 3 tested *Pseudomonas* species in this study. However, a future biological study is recommended to evaluate the immune response of tilapia towards this novel vaccine (mixed with feed) and to determine if this herbal-vaccine combination may show a potent synergistic action between the vaccine (addressing the specific immune response) and the herbal extract (stimulating the non specific immune response).

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