



## The genetic diversity and the antibacterial activity of *Ageratum conyzoides* Linn

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### ABSTRACT

Fourteen samples of *Ageratum conyzoides* cultivated at different places in the Mekong Delta were collected and used for analyzing genetic diversity employing Random Amplified Polymorphic DNA and Inter Simple Sequence Repeat markers and testing the antibacterial susceptibilities expressed as minimum inhibitory concentrations (MIC) of eight selected gram positive and gram negative strains including *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *Aeromonas hydrophila*, *Edwardsiella ictaluri* and *Edwardsiella tarda*. The results showed that *Ageratum conyzoides* had DNA genetic diversity and were divided into 3 groups. All of them had good antibacterial activities against tested bacteria ( $128 \mu\text{g/ml} \leq \text{MIC} \leq 4096 \mu\text{g/ml}$ ), especially against *Edwardsiella ictaluri* and *Edwardsiella tarda* ( $128 \mu\text{g/ml} \leq \text{MIC} \leq 512 \mu\text{g/ml}$ ) and *Staphylococcus aureus* ( $512 \mu\text{g/ml} \leq \text{MIC} \leq 1024 \mu\text{g/ml}$ ).

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## 1 INTRODUCTION

Medicinal plants constitute a valid source of both traditional and modern medicines. *Ageratum conyzoides* is an annual herb, distributed over the tropical and subtropical regions of the world, and widely utilized in traditional medicine wherever it grows (Bioka *et al.*, 1993). A wide range of chemical compounds including alkaloids, flavonoids, chromenes, benzofurans, terpenoids, kaempferol, glycoside (rhamnoside), quercetin, scutellarein, eupalestin, stigmas-7-en-3-ol, sitosterol, stigmasterol, fumaric acid, caffeic acid, saponin, pyrrolizidinic alkaloids, ageratochromene derivatives, and alkane have been isolated from this species (Sharma and Sharma, 2001; Okunade, 2002; Nyuna *et al.*, 2010). Extracts and metabolites from this plant have also been demonstrated antimicrobial, analgesic, antipyretic and anticonvulsant activities (Osho and Adetunji, 2011). As claimed in the folklore

literature, *A. conyzoides* cured various diseases such as constipation, fever, skin ulcers, wounds, dysentery, nephrolithiasis, pneumonia, and burns and was also utilized to treat rheumatism, headache and colic (Vaidyaratnam, 2002).

However, there is no information on the genetic diversity of this plant; furthermore, it is interested in their antibacterial activities against bacteria causing diseases, not only for humans but also for animals. This research, therefore, is aimed to evaluate the genetic diversity of *A. conyzoides* and select the variety having the best antibacterial activity with an expectation that it could take part of the antibiotic role in the future.

## 2 MATERIALS AND METHODS

### 2.1 Materials

A total of 14 samples of *A. conyzoides* cultivated at different places in the Mekong Delta (Can Tho,

Soc Trang, Vinh Long, Bac Lieu, Tra Vinh, Ben Tre, Kien Giang, Dong Thap, Hau Giang, and Ca Mau) were collected for analyzing genetic diversity employing random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. After getting the results of electrophoresis, the *A. conyzoides* plants having genetic dissimilarity were cultivated in Can Tho, in the same conditions of taking care. After 4 months, their leaves were collected for testing of antibacterial susceptibilities expressed as minimum inhibitory concentrations (MIC).

Bacterial strains include *Staphylococcus aureus* 081008 (*S. aureus*), *Streptococcus faecalis* 010408 (*S. faecalis*), *Escherichia coli* 101008 (*E. coli*), *Pseudomonas aeruginosa* 111008 (*P. aeruginosa*), *Salmonella* spp. 291003 (*Sal. spp*), *Edwardsiella tarda* 280208 (*E. tarda*), *Aeromonas hydrophila* 011004 (*A. hydrophila*) from Pasteur Institute (Ho Chi Minh City), and *Edwardsiella ictaluri* CFA 258 – An Giang, 2006 (*E. ictaluri*) from College of Aquaculture and Fisheries (Can Tho University).

**2.2 Methods**

**2.2.1 Genetic diversity**

Genomic DNA of *A. conyzoides* cultivars was extracted using the cetyltrimethylammonium bromide method (Doyle, 1991).

RAPD and ISSR analysis was done using 20 random primers (from First BASE company, Malaysia) in about 10 bases long. The sequences of primers were present in Table 1.

The banding patterns generated by all the different primers were scored as the presence (1) or absence (0) of bands of a particular molecular size to compile to a binary matrix that was then subjected to cluster analysis.

The Statistica 5.5 software was used to analyze the data. Dendrogram from cluster analysis based on unweighted pair-group method with arithmetic mean (UPGMA) of RAPD and ISSR bands was constructed to infer the relationship between the 14 samples of *A. conyzoides* (Sneath and Sokal, 1973).

**Table 1: List of 20 primers used in RAPD and ISSR analysis**

Nº	RAPD primer	Primer sequences (5'.....3')	Nº	ISSR primer	Primer sequences (5'.....3')
1	OPI01	ACCTGGACAC	1	ISSR1	ACACACACACAAG
2	OPI02	GAGGAGAGG	2	ISSR2	AGTGATTGAGTG
3	OPI03	CAGAAGCCCA	3	ISSR3	ACGGACAGACAGACA
4	OPI04	CCGCCTAGTC	4	ISSR5	CTCTCTCTCTCTTG
5	OPI05	TGTTCCACGG	5	ISSR6	GAGAGAGAGAGAGAGAC
6	OPI06	AGGCGGCAG	6	ISSR8	GAGAGAGAGAGAGAGAT
7	OPI07	CAGCGACAAG	7	ISSR10	CACACACACACACACG
8	OPI08	TTTGCCCGGT	8	ISSR11	CACCACCACGC
9	OPI09	TGGAGAGCAG	9	ISSR14	AGCAGCAGCAGCGT
10	OPI10	ACAACGCGAG	10	ISSR15	TCCTCCTCCTCTCC

**2.2.2 Antibacterial activity test**

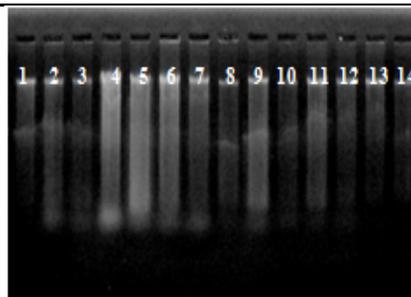
The leaves of *A. conyzoides* were extracted by methanol to get extracts used for testing their antibacterial susceptibility (Nguyen Van Dan and Nguyen Viet Tuu, 1985).

Agar dilution method was used for testing the MIC (Tu Minh Koong , 2007).

**3 RESULTS AND DISCUSSION**

**3.1 Genetic diversity**

After DNA extraction, DNA was quantified by running it on agarose gel 1%. After electrophoresis, the gels were stained with ethidium bromide, and the patterns were visualized intensely under UV light and photographed. The result was shown in Figure 1.



**Fig. 1: The result of electrophoresis quantifying DNA extraction of *A. conyzoides***

In order to evaluate the genetic diversity, 20 primers were used in which 3 RAPD primers and 8 ISSR primers were present in all *A. conyzoides* cultivars tested, and primer ISSR1 produced the most bands with 15 bands amplified for identification. The markers generated 92 amplification products (30 of RAPD markers and 62 of ISSR mark-

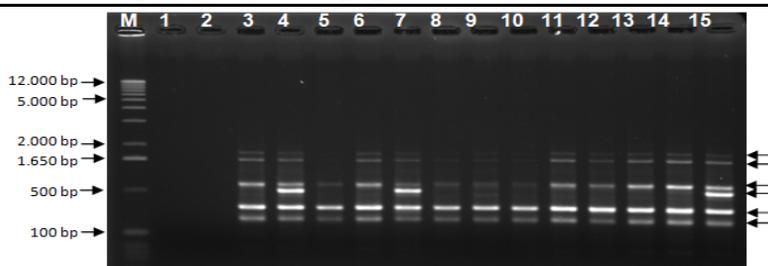
ers) of which 55 bands were polymorphic (59.78%) with  $6.11 \pm 2.72$  polymorphic bands per primer on average. The primer ISSR11 (Figure 2) and ISSR14 (Figure 3) showed the highest rate of polymorphic bands (100%) ranging from 200 - 1800 bp and 500 - 2000 bp, respectively, and the lowest

in primer OPI06 (Figure 4) (15.4%) ranging from 1100 - 2000 bp (Table 2).

The rate of polymorphism in tested cultivars was relatively high (59.78%) indicating a genetic variation among *A. conyzoides* plants in natural conditions.

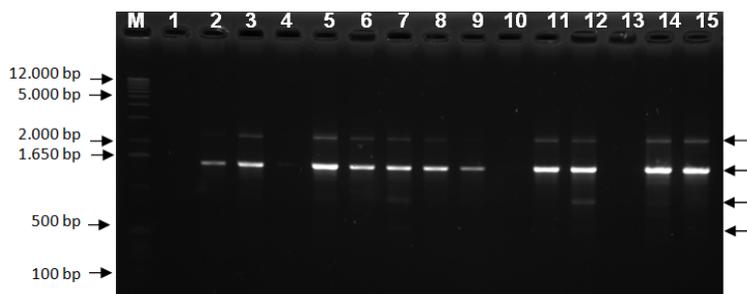
**Table 2: The polymorphisms of 14 *A. conyzoides* cultivars in RAPD and ISSR markers analysis**

No	Primer	Σ DNA bands	Σ polymorphic bands	Ratio (%)	Location of polymorphic bands
1	OPI03	9	4	44.4	1, 2, 8, 9
2	OPI06	13	2	15.4	1, 7
3	OPI07	8	3	37.5	2, 7, 8
4	ISSR1	15	11	73.3	1, 2, 3, 4, 5, 6, 7, 9, 10, 12, 15
5	ISSR3	5	3	60.0	1, 2, 4
6	ISSR6	8	5	62.5	1, 2, 3, 7, 8
7	ISSR8	7	4	57.1	1, 2, 3, 5
8	ISSR10	10	9	90.0	1, 2, 3, 4, 6, 7, 8, 9, 10
9	ISSR11	6	6	100.0	1, 2, 3, 4, 5, 6
10	ISSR14	4	4	100.0	1, 2, 3, 4
11	ISSR15	7	4	57.1	1, 2, 3, 5
Total		92	55		
Average		10.22	6.11	59.78	
± SD		3.29	2.72		



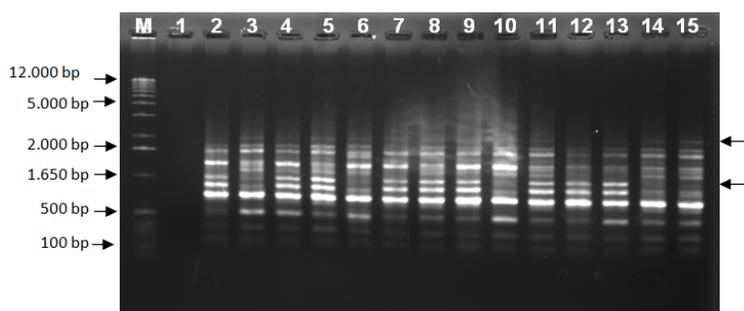
**Fig. 2: Banding patterns obtained using primer ISSR11**

*M: ladder 1 Kb plus (Invitrogen), 1: water, 2...15: cultivar number, ←: polymorphic bands*



**Fig. 3: Banding patterns obtained using primer ISSR14**

*M: ladder 1 Kb plus (Invitrogen), 1: water, 2...15: cultivar number, ←: polymorphic bands*



**Fig. 4: Banding patterns obtained using primer OPI06**

*M: ladder 1 Kb plus (Invitrogen), 1: water, 2...15: cultivar number, ←: polymorphic bands*

With UPGMA cluster analysis, a dendrogram was constructed to infer the relationship between the 14 *A. conyzoides* cultivars that were divided into 3 distinct groups (Figure 5):

Group I: cultivar 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14, showing close relationship with the genetic distance ranging from 2.646 to 4.583.

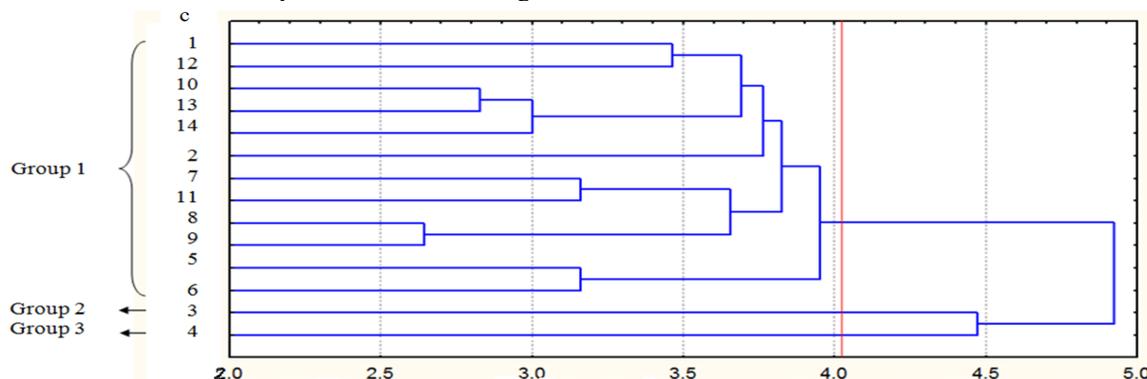
Group II: only cultivar 3

Group III: only cultivar 4

The results from RAPD and ISSR marker analysis showed that tested *A. conyzoides* cultivars had ge-

netic diversities with genetic distance from 2.646 to 5.568. Among them, cultivar 8 and 9 showed the closest relationship with the genetic distance of 2.646 while cultivar 3 showed the farthest relationship to cultivar 1 and 11 with the genetic distance of 5.568.

The high degree of genetic variation could be due to the different geographical locations from which the cultivars were obtained or it could indicate that the cultivars may have to adapt readily to the changes in environmental conditions.



**Fig. 5: Dendrogram from UPGMA analysis using Euclidean distance based on RAPD and ISSR bands of *A. conyzoides***

*The symbol c stands for cultivar*

### 3.2 Antibacterial activity test

The results of testing the antibacterial susceptibilities of tested cultivars were presented in Table 3. It is shown that all *A. conyzoides* groups have antimicrobial efficacy.

Similarly, Kelly *et al.* (2013) observed that the methanolic extract of *A. conyzoides* showed the highest antibacterial activity with the zone diameter of inhibition of 17 mm against *S. aureus*, and the lower against *E. coli* and *P. aeruginosa*. The antibacterial activity of *A. conyzoides* groups against tested bacteria was different and so was the

one in the same group. Group III showed the best inhibitory activity to the bacteria. In general, *A. conyzoides* had antibacterial activities against tested bacteria which cause prevalent diseases to humans and animals and resist a lot of potent antibiotics. Specially, all cultivars have potent activities against *E. ictaluri* and *E. tarda* ( $128 \mu\text{g/ml} \leq \text{MIC} \leq 512 \mu\text{g/ml}$ ) which cause edwardsiellosis in freshwater and marine fishes. *E. ictaluri* causes enteric septicemia, and *E. tarda* causes emphysematous putrefactive disease of catfish and fish gangrene in various species; these diseases have considerable economic losses in the aquacul-

ture industry. *E. tarda* is also an important zoonotic pathogen (Mainous *et al.*, 2010). Besides causing gastroenteritis, the most common disease associated with *E. tarda*, *E. tarda* also causes endocarditis, emphysema, hepatobiliary infection, peritonitis, intra-abdominal abscess, osteomyelitis, wound infection, septicemia, bacteremia, urosepsis, and meningitis in human. The mortality rate of 40-50% has been reported in patients with bacteremia due to *E. tarda* infections (Wang *et al.*, 2005). In addition, *E. tarda* was found to be widely resistant to ampicillin, amoxicillin, cephalixin and erythromycin; resistant to oxytetracycline, tetracycline, nalidixic acid, and sulfonamides more common than to chloramphenicol, florfenicol, ceftiofur, cephalothin, cefoperazone, gentamicin, oxolinic acid, kanamycin and trimethoprim (Akinbowale *et al.*, 2006). According to Spencer *et al.* (2008), Nadirah *et al.* (2012) and Lee *et al.* (2013), most *E. tarda* strains were resistant to colistin, polymixin B, oxacillin, rifampin, fusidic acid, penicillin, oleanomycin, spiramycin, oxolinic acid, ampicillin, erythromycin, amoxicillin, florfenicol, sulfamethoxazole, chloramphenicol, nalidixic acid, doxycycline, flumequine, kanamycin, novobiocin, tetracycline, fosfomicin, and lincomycin. Similarly to *E. tarda*, *E. ictaluri* has also widely resisted a lot of potent antibiotics (Tu Thanh Dung *et al.*, 2008). *A. conyzoides* also have good inhibitory activity against *S. aureus* which is responsible for many infections including bacteremia leading to formation of multiple abscesses in joints, liver, kidney, umbilicus, spleen or lymph nodes to give osteomyelitis; vegetative endocarditis and also causing infarction in the kidney, mastitis, vaginitis, metritis, and the abortion has been associated with the demonstration of serum antibody to alpha hemolysin in the sow. Its enterotoxin production is possible for causing food poisoning in humans (Taylor, 2006). *S. aureus* now has the high level of resistance to penicillin (89.4%), tetracycline (82.4%), trimethoprim-sulfamethazine (80.6%), chloramphenicol (64.8%), erythromycin (38.4%), and methicillin (35.9%) (Anakalo and Milcah, 2004). It also develops the resistance to antibiotics having a potent antibacterial activity such as ceftriaxol, ciprofloxacin (Le Huy Chinh, 2003). The pathogen showed high resistance against penicillin G (86%) and tetracycline (76.7%), erythromycin (39.5%), clindamycin (34.9%), cefoxitin (16.3%), oxacillin, chloramphenicol, trimethoprim-sulfamethoxazole (11.6%), lincomycin (9.3%), gentamicin (7%), quinupristin-dalfopristin and streptomycin (2.3%) (Hossein *et al.*, 2014).

**Table 3: Antibacterial activity of the extracts of tested cultivars**

Bacteria	MIC (µg/ml)		
	Group I	Group II	Group III
<i>S. aureus</i>	512	1024	512
<i>S. faecalis</i>	4096	4096	1024
<i>E. coli</i>	>4096	2048	2048
<i>P. aeruginosa</i>	4096	2048	2048
<i>Sal. spp.</i>	>4096	4096	4096
<i>A. hydrophila</i>	4096	2048	2048
<i>E. ictaluri</i>	512	128	128
<i>E. tarda</i>	512	128	128

Note: Group I: cultivar 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14; Group II: cultivar 3; Group III: cultivar 4

There are many active compounds having been found in *A. conyzoides* including alkaloids, triterpenoids, almarins, essential oils, tannins, agerchromone, 2, 6-dimefloyageratochromone, eugenol and flavonoids such as conyzoigun, and dotriacanthene which could be responsible for their antimicrobial effects. 2,2-dimethyl chromene derivatives like 6(1-hydroxyethyl)-7,8-dimethoxy-2,2-dimethylchromene and 6-hydroxy-7,8-dimethoxy-2,2 dimthyl chromene have also been shown to have antimicrobial activities (Gouri and Murthy, 2011; Osho and Adetunji, 2011).

In this study, the cultivars clearly demonstrate antibacterial properties to *E. ictaluri*, *E. tarda* and *S. aureus* (although the mechanistic processes are poorly understood). As bacteria become resistant to more and more antibiotics, many attempts have been made to find other ways to treat infections. The discovery of antibacterial activity of *A. conyzoides* to tested bacteria has an important contribution for further research to produce antibacterial agents from plants that can take the role of chemical antibiotics in preventing and treating diseases of animals.

#### 4 CONCLUSIONS

Basing on RAPD and ISSR markers, *A. conyzoides* was divided into 3 distinct groups. The antibacterial activities were different among 3 groups which exhibited the best inhibition against *E. ictaluri*, *E. tarda* and *S. aureus*.

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