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Antimicrobial properties of medicinal herbal extracts against pathogenic bacteria isolated from the infected grouper *Epinephelus tauvina*

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A study was undertaken to investigate the antimicrobial properties of herbal extracts against bacterial pathogens isolated from infected grouper *Epinephelus tauvina*. The tissue and blood samples were aseptically collected from the infected grouper and the bacterial diversity was screened and identified up to species level. From the identified pathogens, bacterial species such as *Streptococcus pyogenes*, *Staphylococcus aureus*, *Serratia salinaria*, *Alcaligen faecalis* and *Vibrio parahaemolyticus* were selected for sensitivity study on the herbal compounds extracted from *Withania somnifera*, *Tinospora cordifolia*, *Solanum xanthocarpum*, *Daemia extensa* and *Andrographis paniculata*. The bacterial growth inhibitory effect was found to be dependant on the solvent used for extraction as well as specific to herbal extracts. The minimum inhibitory concentrations of selected herbal extracts were 50 ppm for *W. somnifera* in benzene on *A. faecalis*, 12.5 ppm for *T. cordifolia* in propanol on *V. parahaemolyticus* and 100 ppm for *S. xanthocarpum* in petroleum ether on *S. salinaria*. The study was also extended to find out the inhibitory effect of phenolic compounds of herbal extracts on bacterial pathogens and only *W. somnifera* showed a positive effect. The thin layer chromatography results showed that alkaloids such as piperine, concessine, codeine and morphine-like compounds may be responsible for the inhibitory effect of herbal products from *T. cordifolia, S. xanthocarpum* and *A. paniculata*.

Key words: herbal extracts, *Epinephelus tauvina*, *Withania somnifera*, *Tinospora cordifolia*, *Solanum xanthocarpum*, *Daemia extensa*, *Andrographis paniculata*.

INTRODUCTION

Groupers are being cultured worldwide, especially in Asian countries in floating net cages and the important species include *Epinephelus coioides*, *E. tauvina*, *E. fuscoguttatus*, *E. lanceolatus*, *Plectropomus leopardus* and *Cromleptes altivelis*. Until recently, grouper production has been relatively high, but nowadays, the production has dropped drastically due to stresses, cannibalism, environmental problems associated with poor water quality and also disease outbreaks (Sadovy, 2000). It has been also reported that the survival rate of *E. lanceolatus* and *C. altivelis* during culture conditions was low, since they are very often susceptible to disease. Unfortunately, knowledge on disease outbreaks and the remedial measures pertaining to grouper culture is very limited (Chan, 2000).

In groupers, Gram negative bacteria, especially *Vibrio parahaemolyticus* and *V. alginolyticus*, cause hemorrhagic septicemia, particularly in nursery and grow-out cage systems. The Gram positive bacterium *Streptococcus* sp. also causes systemic infection in grouper. Additional factors such as nutritional deficiency and environmental stresses also cause health problems in cultured fishes. Therefore, there is a great urgency to find ways to reduce mortality in grouper culture. One way of achieving this is by vaccination. Vaccination of groupers against bacterial disease is very important during their culture period. Vaccination strategies for disease management are partially successful, but proper vaccination protocols are yet to be developed (Ong & Wong, 1988). In Asian regions, various antimicrobials are used in fish

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and shrimp production, to control deadly infectious diseases caused by a variety of pathogenic bacteria. Also, chemotherapeutic agents like antibiotics, disinfectants and non-specific synthetic immuno-stimulants are commonly employed for disease management in small- scale culture systems. However, in the case of large-scale operations, it is not advisable because of the cost effectiveness and the potential development of drug resistance against antibiotics due to improper administration practices (Kruse & Soram, 1994). Furthermore, the use of probiotics in disease management is also at experimental phase in aquaculture industries.

In this scenario, plant-based antimicrobials remain a vast untapped source for medicines with enormous therapeutic potential. They are effective in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Several attempts have been made earlier to develop drugs against pathogens and they can also be applied in aquaculture practices to eradicate pathogens from the host (Press, 1996; Lawrence, 1999; Immanuel *et al*., 2004). Hence, herbal products are promising to be an important source for therapeutics and may be a viable solution for disease problems in fish culture, especially grouper culture.

The search for biologically active extracts based on traditionally used plants is still relevant and has been highlighted by Leaman *et al*. (1995). This approach has also led to the discovery of sources rich in compounds with antibiotic and antibacterial activities (Vlietinck & Vanden Berghe, 1991; Yip *et al*., 1991; Hudson, 1995).

Phytomedicines usually have multiple effects, their actions often going beyond the symptomatic treatment of disease (Murray, 1995). Chemotherapeutic agents, which are isolated from plants, have been proven effective against drug resistant bacteria (Richard & Anthony, 1983). Compounds such as volatile oils, phenolic compounds including tannins, saponins, alkaloids, polysaccharides and polypeptides were shown to be effective alternatives to antibiotics (Direkbusarakom *et al*., 1995, 1997). Literature related to disease problems due to microbes and possible remedial measures for grouper is scanty, hence the present study was undertaken to screen herbal extracts for the antagonistic effect against grouper pathogens.

MATERIALS AND METHODS

For the present study, infected grouper *Epinephelus tauvina* (n = 5) were collected from the fish landing centre at Kadiyapattanam, K.K. District, Tamilnadu, India. The collected fish samples were transported immediately under aseptic conditions to the laboratory and muscle samples were collected from the infected area. Simultaneously, blood and mucus samples were also collected using a sterile hypodermal syringe and a sterile scalpel for bacteriological examinations.

Isolation and characterization of pathogens

The muscle tissue samples were homogenized with alkaline peptone water (Hi Media) and 3% NaCl (APW 3%). The blood and mucus samples were transferred separately to 3% APW in 500 ml conical flasks and incubated at 37°C for enrichment. One gram of tissue sample was taken, homogenized and a series of solutions were made at 10^{-2}-10^{-7} concentrations. Then, 1 ml of each solution was inoculated in Zobel marine agar (Hi Media) plates by both spread- and pour-plating methods and incubated for 24 h. Likewise, 1 ml of serial solution (10^{-2}-10^{-7}) was also made for blood and mucus swabs, inoculated in the same agar medium and incubated. Plates with colony numbers ranging from 50 to 300 were taken for enumeration. Using colony morphology, shape and color, the major dominant microflora was isolated and again streaked on Zobel marine agar plates. Pure cultures were isolated from these plates and slants were made. Further identification up to species level was done based on the rapid slide agglutination method of serological typing (ready made sera used, ZYDUS Pathline Ltd., Mumbai, India), as well as on biochemical and physiological tests of isolates according to standard procedures (Holt *et al*., 1996).

Preparation of herbal powder from medicinal plant materials

Five different medicinal herbs, i.e. *Withania somnifera*, *Tinospora cordifolia*, *Solanum xanthocarpum*, *Daemia extensa* and *Andrographis paniculata* were selected for the present study. Sufficient quantities of these herbs were collected from various regions of the Kanyakumari District, India. The collected herbal plants were washed thoroughly to remove dust and other adherent materials and dried under shade at room temperature. The dried plants were ground well and

sieved in order to remove plant fibres. The larger particles were ground again and sieved through a fine cloth (mesh size < 50 mm) to obtain a product with uniform particle size.

Preparation of herbal extracts

For the present experiment, selected herbal powders were individually mixed with five different solvents, i.e. n-butanol, propanol, petroleum ether, acetone and benzene. Extraction was done by soaking one part of the product (powder form) into three parts of liquid solvent (1:3) and the mixtures were kept for the infusion process for three days. The crude extracts were then filtered and the filtrates were concentrated to dryness by allowing them for complete evaporation of solvent under room temperature. From this stock extract, various experimental concentrations were prepared with the respective solvents.

Sterilized discs were soaked in the prepared herbal extracts of different concentrations and kept overnight at room temperature. The soaked discs were then dried aseptically to ensure evaporation of the solvents. The Muller-Hinton agar medium was prepared using sterilized seawater and poured in each Petri dish and allowed to cool. Swabs were prepared from various stock cultures of pathogens, and spread over the agar surface with a cotton swab to make a lawn. The plates were allowed to dry for 20 min. Dried antimicrobial discs with impregnated herbal extracts and control discs (without antimicrobials) were carefully dispensed at uniform distances over the agar surface and ensured for correct implantation by applying gentle pressure over the discs. All plates were kept for incubation at 35°C for 24 h. After incubation, plates were observed for inhibitory zone formation of the antimicrobial extracts on the microbial lawns.

Minimum inhibitory concentration (MIC)

The MIC is defined as the lowest antimicrobial concentration of the test compounds, which inhibits bacterial growth. To assess MIC, the stock herbal extracts were solubilised in Muller-Hinton's broth (MHB) and serially diluted in the same broth to obtain desired concentrations such as 100, 50, 25, 12.5, 6.5, 3.125, 1.563 and 0 (control) ppm. Samples measuring 80 ml of each bacterial suspension were added to the serially diluted test substances. The MHB without plant extract was used as control. The inoculated test tubes were incubated at 35°C for 24 h. After the incubation period, turbidity was observed. The test tubes, which did not show any turbidity due to suppressing of microbial growth, indicated the presence of active antibiotic at a concentration corresponding to MIC.

Test for lipophilic phenolic compounds

Twenty-five g of herbal powder was mixed with 95% toluene-methanol mixture (1:3 v/v) and kept at room temperature for infusion. The extracts were filtered through Whatman No1 filter paper and taken in a separating funnel. Then, 1-3 ml of 1N aqueous sodium nitrate was added (30-60 min) and the upper layer was collected. Sterilized paper discs were soaked into it and kept overnight at room temperature. Control discs were also prepared by soaking in toluene-methanol mixture alone. Then the soaked discs were aseptically dried to ensure evaporation of the solvents.

The inhibitory effect of the lipophilic compounds in the selected herbal products was estimated following the method by Nostro *et al*. (2000) on the identified microbial pathogen.

$$\text{Inhibition } (\%) = \frac{X - D \times 100}{Y}$$

where, X = diameter of the zone, D = disc diameter and Y = maximum inhibition zone.

Identification of alkaloids

The alkaloids present in the selected herbal products were identified by thin layer chromatography (TLC) using two different staining reagents (Edmann and Marquis) as described by Chakraborty *et al*. (1999). To begin with, 0.25 mm thick TLC plates were prepared on glass plates (20 × 10 cm) by using silica gelG. After activation at 100-120°C for 1 h, the samples were loaded at a concentration of 3 ml in the base line, i.e. 3 cm above the bottom. Then, the chromatogram was developed by using a mobile phase containing benzene, ethyl acetate and tri-ethyl amine in a ratio of 6:3:1 to a height of 15 cm. After that, the detection of alkaloids on the developed chromatogram was performed by spraying standard staining solutions for alkaloids. Considering the specific staining characteristics of alkaloids, two different staining solutions were used in the present work and hence, two different chromatograms were developed simul-

taneously for the test samples and were stained separately in Edmann and Marquis reagents. After staining, the positions of the samples in the chromatogram were located and the Rf (Retention factor) values were calculated. By referring the standard Rf values pertinent to the used mobile phase, the alkaloids were identified.

Antibacterial testing by commercial antibiotics

The antibacterial activity of 14 commercial antibiotics (Hi Media) commonly used in aquaculture industries (Table 9) was tested against the isolated bacterial pathogens as described above.

RESULTS

Bacterial diversity

The occurrence of total aerobic heterotrophic bacterial genera was assessed both in tissue and blood samples of infected grouper *E. tauvina*.

TABLE 1. Bacterial composition (%) isolated from infected tissue and blood samples of grouper *Epinephelus tauvina*

Serial No	Bacterial genera	Test samples	
		Tissue	Blood
	Total isolates	13	6
1	*Vibrio*	4 (30.77%)	2 (33.35%)
2	*Corynebacterium*	2 (15.38%)	–
3	*Bacillus*	1 (7.69%)	1 (16.67%)
4	*Flexibacter*	1 (7.69%)	–
5	*Aeromonas*	1 (7.69%)	–
6	*Pseudomonas*	1 (7.69%)	–
7	*Streptococcus*	2 (15.38%)	–
8	*Serratia*	1 (7.69%)	–
9	*Proteus*	–	1 (16.67%)
10	*Alcaligenes*	–	1 (16.67%)
11	*Staphylococcus*	–	1 (16.67%)

TABLE 2. Results on High Titer Serum method (rapid slide agglutination method) of bacterial genera isolated from the infected grouper *Epinephelus tauvina* (+/– = agglutination/non-agglutination)

Serial No	Samples	Pathogens	Antisera used	Dilution	Results
1		*Vibrio*	*V. parahaemolyticus*	1 : 640	+
2		*Corynebacterium*	*Corynebacterium*	1 : 160	–
3		*Bacillus*	*B. licheniformis*	1 : 80	+
4		*Flexibacter*	*Flexibacter*	1 : 160	–
5		*Aeromonas*	*A. hydrophila*	1 : 320	+
6	Tissue	*Pseudomonas*	*P. aeruginosa*	1 : 160	+
7		*Streptococcus*	*S. pyogenes*	1 : 320	+
8		*Streptococcus*	*S. faecium*	1 : 320	+
9		*Vibrio*	*V. parahaemolyticus*	1 : 640	+
10		*Corynebacterium*	*Corynebacterium*	1 : 160	–
11		*Serratia*	*S. salinaria*	1 : 320	+
12		*Vibrio*	*V. parahaemolyticus*	1 : 640	+
13		*Vibrio*	*V. parahaemolyticus*	1 : 640	+
1	Blood	*Vibrio*	*V. parahaemolyticus*	1 : 640	+
2		*Proteus*	*P. mirabilis*	1 : 320	+
3		*Alcaligenes*	*A. faecalis*	1 : 640	+
4		*Staphylococcus*	*S. aureus*	1 : 320	+
5		*Bacillus*	*B. licheniformis*	1 : 80	+
6		*Vibrio*	*V. parahaemolyticus*	1 : 640	+

The results on the number of bacterial genera identified, and their percentage occurrence are provided in Table 1. In total, 19 isolates were selected, i.e. 13 from the infected tissue and six from the blood sample. In the tissue sample, eight bacterial genera were identified. They were: *Vibrio* sp., *Corynebacterium* sp., *Bacillus* sp., *Flexibacter* sp., *Aeromonas* sp., *Pseudomonas* sp., *Streptococcus* sp. and *Serratia* sp. Among these identified bacterial genera, *Vibrio* sp. was the most dominant one with 30.77% occurrence. The next dominant genera were *Corynebacterium* sp. and *Streptococcus* sp. with a percentage occurrence of 15.38%. In the blood sample, of the six total isolates, five bacterial genera such as *Vibrio* sp., *Proteus* sp., *Alcaligenes* sp., *Staphylococcus* sp. and *Bacillus* sp. were identified. Similarly, in the tissue sample, *Vibrio* sp. dominated with 33.35% occurrence.

Confirmation test (high titer serum method)

The identified bacterial genera were subjected to the high titer serum method (rapid slide agglutination method) for further confirmation up to species level. Table 2 shows the results with the high titer serum method of the selected pathogens to respective antisera. Except for *Flexibacter* and *Corynebacterium*, all other microbes showed a positive result with the tested antisera. For instance, the bacterial genus *Vibrio* showed a positive agglutination with *Vibrio para-*

TABLE 3. Zone of inhibition of selected herbal products extracted in different organic solvents against bacterial pathogens isolated from infected grouper *Epinephelus tauvina* (3 replicates, mean ± SD)

Herbal products	Solvents	Microbial species / Zone of inhibition (mm)				
		S.aureus	*A.faecalis*	*S.pyogenes*	*S.salinaria*	*V.parahaemolyticus*
A. paniuculata	benzene	11 ± 0.75	–	11 ± 1.00	–	7 ± 0.50
	n-butanol	–	–	6 ± 0.50	8 ± 0.75	7 ± 0.50
	propanol	–	–	–	11 ± 1.00	8 ± 0.50
	acetone	–	–	12 ± 1.50	–	–
	petroleum ether	–	–	6 ± 0.50	8 ± 0.50	7 ± 0.50
	control	–	–	–	–	–
D. extensa	benzene	–		–	–	–
	n-butanol	8 ± 0.50		9 ± 1.00	–	–
	propanol	–		–	–	–
	acetone	9 ± 0.50		–	–	–
	petroleum ether	–		–	–	–
	control	–		–	–	–
T. cordifolia	benzene	–	–	–	–	–
	n-butanol	7 ± 0.50	–	–	–	–
	propanol	–	–	–	10 ± 1.00	15 ± 1.50
	acetone	10	–	–	–	–
	petroleum ether	–	–	–	8 ± 1.00	–
	control	–	–	–	–	–
S. xanthocarpum	benzene	–	–	–	–	–
	n-butanol	8 ± 1.00	11 ± 1.50	–	10 ± 1.00	7 ± 0.50
	propanol	–	10 ± 1.00	9 ± 0.50	12 ± 1.00	–
	acetone	9 ± 1.00	–	–	–	–
	petroleum ether	–	–	–	14 ± 1.00	–
	control	–	–	–	–	–
W. somnifera	benzene	13 ± 1.00	13 ± 1.50	15 ± 2.00	–	10 ± 0.75
	n-butanol	10 ± 0.50	–	12 ± 1.00	13 ± 1.00	11 ± 0.50
	propanol	9 ± 0.50	9 ± 0.75	8 ± 0.50	10 ± 1.00	7 ± 0.25
	acetone	15 ± 1.50	–	21 ± 2.00	–	12 ± 1.00
	petroleum ether	–	–	–	12 ± 1.00	–
	control	–	–	–	–	–

TABLE 4. Zone of inhibition (mm)of combined action of selected herbal products extracted in acetone and propanol (1:1) against bacterial pathogens isolated from infected grouper *Epinephelus tauvina* (3 replicates, mean ± SD)

Serial No	Herbal products	Microbial species / Zone of inhibition (mm)				
		S. aureus	*S. pyogenes*	*A. faecalis*	*S. salinaria*	*V. parahaemolyticus*
1	*A. paniculata*	7 ± 0.50	7 ± 0.50	–	8 ± 0.75	–
2	*S. xanthocarpum*	–	–	–	–	–
3	*T.cordifolia*	–	–	–	9 ± 1.00	–
4	*W. somnifera*	14 ± 1.50	14 ± 1.00	12 ± 1.00	12 ± 0.75	8 ± 0.50
5	Control	–	–	–	–	–

haemolyticus antisera. This confirms the *Vibrio* bacterial genus isolated as *V. parahaemolyticus*. Similarly, the isolated *Bacillus* sp. exhibited a positive agglutination with *B. licheniformis* antisera, which confirms its identity as *B. licheniformis*. The bacterial genera *Aeromonas*, *Pseudomonas*, *Streptococcus*, *Serratia*, *Staphylococcus*, *Proteus* and *Alcaligenes* showed a positive agglutination with the respective antisera as *A. hydrophila*, *P. aeruginosa*, *S. pyogenes*, *S. faecium*, *S. salinaria*, *S. aureus*, *P. mirabilis* and *A. faecalis* and confirmed their species identity.

Antimicrobial activity of herbal products

Antimicrobial activity of five different herbal products has been tested on different solvents based on their use in ancient medicine. The results on the antimicrobial activity (zone of inhibition) of chosen herbal products (Table 3) showed that *A. paniculata* extracted with benzene, acetone and propanol inhibited *S. pyogenes*, *S. aureus* and *S. salinaria* with an inhibition zone of 11 and 12 mm, respectively. These products however did not show any inhibitory effect on *A. faecalis*. It was also inferred a solvent-dependent inhibitory effect of the selected herbal product. For example, *A. paniculata* extracted with propanol showed a maximum inhibitory effect on *S. salinaria*, whereas it showed an inhibitory action only on *S. pyogenes* when extracted with acetone.

The inhibitory effect of *D. extensa* extracted with selected solvents on the isolated bacterial species was not remarkable. Here, the zone of inhibition was observed only in *S. aureus* and *S. pyogenes* when extracted with butanol and acetone.

Viewing the magnitude of inhibitory zone, the herbal products such as *T. cordifolia* and *S. xanthocarpum* displayed a moderate inhibitory effect. *Tinospora cordifolia* showed an inhibitory effect on three bacterial pathogens with the inhibition zone ranging from 7.0 to 15.0 mm. On the other hand, *T. cordifolia* did not show any inhibitory effect when extracted with benzene. In this particular case, the maximum zone of inhibition was recorded when extracted with propanol and acetone. Likewise, *S. xanthocarpum* showed a zone of inhibition in all the isolated pathogens with the inhibition zone ranging from 7.0 to 14.0 mm. In this case, a maximum zone of inhibition was registered in *S. salinaria* extracted with propanol (12.0 mm) and petroleum ether (14.0 mm). However, *S. xanthocarpum* did not show any inhibitory effect when extracted with benzene.

More promising results were obtained in *W. somnifera* extracted with tested organic solvents against all the isolated microbial pathogens. In this product, the maximum zones of inhibition (21.0 and 15.0 mm) were noticed respectively on *S. pyogenes* and *S. aureus* when extracted with acetone. While extracted with benzene and butanol, *W. somnifera* showed an inhibitory effect against the majority of the pathogens isolated and it ranged from 10.0 to 15.0 mm and from 10.0 to 13.0 mm, respectively (Table 3).

The results on the combined effect of acetone and propanol in a ratio of 1:1 on the inhibitory action of selected herbal products indicated that, *W. somnifera* showed a maximum inhibitory effect on all the isolated pathogens with the inhibitory zone ranging from 8.0 to 14.0 mm. By the same manner, *A. paniculata* showed an inhibitory effect against *S. aureus*, *S. pyogenes* and *S. salinaria*, whereas *S. xanthocarpum* did not show any inhibitory effect (Table 4).

MIC of selected herbal products

Minimum inhibitory concentration (MIC) was assessed for all the selected herbal extracts on the isolated bacterial pathogens by giving due consideration to bacterial growth inhibition by the respective herbal products (Table 5). Accordingly, the MIC of *W. som-*

TABLE 5. Minimum inhibitory concentration (MIC) of selected herbal extracts at the maximum inhibition solvents on bacterial pathogens isolated from infected grouper *Epinephelus tauvina*

Pathogens / herbal products	MIC (ppm)
A. faecalis / *W. somnifera*	50
V. parahaemolyticus / *T. cordifolia*	50
S. pyogenes / *W. somnifera*	12.5
S. aureus / *W. somnifera*	12.5
S. salinaria / *S. xanthocarpum*	100

nifera was 50 ppm on *A. faecalis* and 12.5 ppm on both *S. pyogenes* and *S. aureus*. Likewise, the MIC of *T. cordifolia* on *V. parahaemolyticus* was 50.0 ppm. The MIC of *S. xanthocarpum* on *S. salinaria* was at the maximum concentration of 100 ppm.

Inhibitory effect of phenolic compounds

Data on sensitivity and percentage inhibition of phenolic compounds extracted from the selected herbal products are given in Table 6. Out of five herbal extracts tested, positive sensitivity result was obtained only for *W. somnifera* and no inhibitory zone was noticed for all other four tested herbal products. The zone of inhibition of phenolic compounds on selected bacterial pathogens varied from 6.0 to 8.0 mm and the percentage inhibition ranged from 12.5 to 37.5%, being the maximum at *S. salinaria*.

TLC separation of alkaloids

Table 7 provides the qualitative analysis of alkaloids in selected herbal products with two different staining reagents (Edmann and Marquis). Altogether, four alkaloids were identified. They were: piperine, morphine-like compound, concessine and codeine. These alkaloids were present in *T. cordifolia*, *S. xanthocarpum* and *S. paniculata*. An unknown alkaloid with an Rf value of 0.98 was also identified in all tested herbal products.

TABLE 6. Zone of inhibition (mm) of phenolic compounds from the selected herbal products against the pathogenic bacteria isolated from infected grouper *Epinephelus tauvina*. Values in parentheses are the % inhibition (3 replicates, mean ± SD)

Serial No	Herbal products	*S. aureus*	*S. pyogenes*	*A. faecalis*	*S. salinaria*	*V. parahaemolyticus*
1	*T. cordifolia*	–	–	–	–	–
2	*S. xanthocarpum*	–	–	–	–	–
3	*D. extensa*	–	–	–	–	–
4	*W. somnifera*	6 ± 0.50 (12.5)	7 ± 0.75 (25)	6 ± 0.40 (12.5)	8 ± 1.00 (37.5)	7 ± 0.50 (25)
5	*A. paniculata*	–	–	–	–	–
6	Control	–	–	–	–	–

TABLE 7. Thin layer chromatography separation of alkaloids present in the selected herbal products in two different staining reagents (* = morphine-like compound, +/– = presence/absence of alkaloids)

Herbal products	Rf value	Alkaloid	Reagent	
			Edmann	Marquis
W. somnifera	0.98	unknown	+	–
	0.06	unknown	+	–
T. cordifolia	0.25	piperine	+	–
	0.98	unknown	+	+
	0.10	morphine*	+	–
S. xanthocarpum	0.98	unknown	+	+
	0.80	concessine	–	+
	0.10	morphine*	+	+
	0.38	codeine	+	+
A. paniculata	0.80	concessine	+	+
	0.98	unknown	+	+

TABLE 8. Antibiotic (commercial) sensitivity test on bacterial pathogens isolated from the infected grouper *Epinephelus tauvina* (R = resistant, nt = not tested; 3 replicates, mean ± SD)

Antibiotics	Pathogens / Zone of inhibition (mm)				
	S. salinaria	*S. aureus*	*A. faecalis*	*S. pyogenes*	*V. parahaemolyticus*
Gentamicin	25 ± 2.0	28 ± 2.5	26 ± 1.0	22 ± 1.0	R
Nitrofurantion	25 ± 2.0	R	20 ± 1.0	R	22 ± 2.0
Norfloxacin	25 ± 3.0	28 ± 3.0	28 ± 2.0	24 ± 2.0	22 ± 1.0
Pencillin G	R	20 ± 1.0	R	R	R
Amikacin	25 ± 2.5	24 ± 1.5	28 ± 2.0	22 ± 1.0	nt
Novobiocin	20 ± 1.5	12 ± 1.0	R	10 ± 1.0	nt
Colistin	15 ± 1.5	12 ± 1.0	10 ± 0.5	12 ± 1.0	nt
Kanamycin	10 ± 1.0	20 ± 2.0	12 ± 1.0	18 ± 2.0	nt
Cefotaxime	R	R	26 ± 1.0	R	nt
Lomefloxacin	R	28 ± 3.0	28 ± 2.5	24 ± 2.0	nt
Tobramycin	R	24 ± 2.0	24 ± 2.0	22 ± 1.5	nt
Cephalexin	R	12 ± 1.0	R	12 ± 0.5	nt
Netilmicin	R	20 ± 2.0	24 ± 2.0	18 ± 1.5	nt
Cefuroxime	R	18 ± 1.5	12 ± 0.5	R	nt

Antibiotic (commercial) response of isolated pathogens

In the present study, the antibiotic assay was also performed on the isolated pathogens and the results are provided in Table 8. Due to technical reasons, the sensitivity test for *V. parahaemolyticus* was made only on four antibiotics (gentamicin, nitrofurantion, norfloxacin and penicillin G) out of 14 antibiotics selected. For the remaining four bacterial pathogens (*S. salinaria*, *S. aureus*, *A. faecalis* and *S. pyogenes*), the sensitivity test was carried out in all the 14 antibiotics. The results on sensitivity test indicated that, *V. parahaemolyticus* was resistant to gentamicin and penicillin G. The bacterial pathogen *S. salinaria* was sensitive to gentamicin, nitrofurantion, norfloxacin, amikacin, novobiocin, colistin and kanamycin and resistant to all the other tested antibiotics. The pathogen *S. aureus* was resistant to only two antibiotics (nitrofurantion and cefotaxime) and sensitive to all the other 12 antibiotics with a zone of inhibition ranging from 12.0 to 28.0 mm. Similarly, *S. pyogenes* was originally resistant to four antibiotics, i.e. nitrofurantion, penicillin G, cefotaxime and cefuroxime and sensitive (10.0 to 24.0 mm) to the other tested antibiotics. The pathogen *A. faecalis* was resistant to penicillin G, novobiocin and cephalexin and sensitive to all the other tested antibiotics.

DISCUSSION

The present results demonstrated that the composition and percentage occurrence of bacterial pathogens was relatively higher in the tissue sample than the blood sample of the infected grouper. The results on rapid slide agglutination test indicated the occurrence of eight bacterial genera in the tissue sample and six bacterial genera in the blood sample. The results on the microbial growth inhibitory effect of the herbal extracts on the five selected dominant bacterial genera implied the existence of an antimicrobial potency and it was found to be solvent- dependent.

Irrespective of the solvent systems, the selected herbal extract showed an inhibition zone ranging from 13.0 to 21.0 mm on the tested bacterial pathogens. *Withania somnifera* showed a maximum inhibition effect on *S. aureus* and *S. pyogenes* in acetone extract and on *A. faecalis* in benzene extract. *Solanum xanthocarpum* and *T. cordifolia* showed an antimicrobial potency when extracted in petroleum ether and propanol, respectively on *S. salinaria* and *V. parahaemolyticus*. From this, it may be inferred that these herbal products have a potent antimicrobial activity to control bacterial pathogens. A similar result was also reported by Cos *et al*. (2002). In their study, they evaluated Rwandan medicinal plant extracts for their antimicrobial and antiviral activities. In total, 45

Rwandan plant extracts belonging to 37 different plant species in 21 families were tested. From all the plant extracts tested, only that of *Clematis hirsuta* (leaves) showed a pronounced antifungal activity against *Candida albicans* and the dermatophytes *Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum canis*. Few of the plant extracts showed high antiviral activity against the DNA virus *Herpes simplex* type l.

The results of the minimum inhibitory effect on the selected bacterial pathogens at the respective inhibitory solvent revealed that, a concentration of 12.5 ppm *W. somnifera* extracted with acetone was found to the MIC on *S. pyogenes* and *S. aureus*. The same compound showed a MIC at 50.0 ppm on *A. faecalis* when extracted with benzene. A MIC of 50.0 ppm was also noticed in the extract of *T. cordifolia* on *V. parahaemolyticus* when extracted with propanol. A low MIC value of 100 ppm was noticed in the extract of *S. xanthocarpum* with petroleum ether on *S. salinaria*. Okeke *et al*. (2001), when evaluating the extracts of the root of *Landolphia owerrience* reported that, the antibacterial activity of root extracts varied with the kind of solvents used for the extraction. Crude preparations of whole plant parts containing both the active and non-active components have been suggested to have a higher efficiency than semi-crude or pure plant substances (Kafaru, 1994).

In a study on the antibacterial activity of extracts from some edible plants commonly consumed in Asia, Alzoreky & Nakahara (2002) reported that the buffered methanol and acetone were proved to be good solvents in extracting inhibitory substances from the plant materials. This result is consistent with the present finding that *W. somnifera* extracted with acetone showed a better inhibitory effect on two out of five bacterial pathogens tested. In contrast, Eloff (1998) and Cowan (1999) found that methanol was more efficient than acetone in extracting phytochemicals from plant materials. Otshudi *et al*. (1999) considered that diethyl ether extracts of plants were inactive against bacteria compared with aqueous methanol extracts. It was reported that plants having microbial inhibitors (i.e., flavonoids) soluble in aqueous methanol, and the flavonoid aglycones were more active than their glycosidic forms naturally present in plants (Otshudi *et al*., 1999; Rauha *et al*., 2000). This may partly explain the broad inhibitory activity of naringenin and quercetin compounds compared with the crude extract of the plants (Alzoreky & Nakahara, 2002).

In the present study, the effects of commercially available antibiotics were determined on the selected bacterial pathogens. The results showed that *S. pyogenes* has developed a resistance for four commercial antibiotics among the 14 tested. Other pathogens also exhibited a resistance against commercial antibiotics. Despite this resistance, development of *S. aureus* and *S. pyogenes* was successfully inhibited by the extracts of *W. somnifera*. This paves the way for further investigation on herbal products to screen the compounds responsible for the antimicrobial activity. In the present study, phenolic compound inhibitory effect was positive only in *W. somnifera* on the tested bacterial pathogens with a percentage inhibition of 12.5 to 37.50%. In other products, the alkaloids piperine, a morphine-like compound, concessine and codeine were also screened by the TLC. From this, it may be inferred that the antibacterial activity shown by the selected herbal compounds may be due to the presence of phenolic compounds in *W. somnifera* and alkaloids in other herbal products such as *T. cordifolia* (piperine), *S. xanthocarpum* (a morphine-like compound and concessine) and *A. paniculata* (a morphine-like compound, codeine and concessine). Chakraborty *et al*. (1999) have also reported a similar view when studying the antibacterial steroid alkaloids of the stem bark of *Holarrhena pubescens*. In their observations, the crude methanolic extracts exhibited the antibacterial activity to different degrees (10 to 16 mm). They also pointed out that the alkaloid fraction showed a significant activity against *S. aureus*, *S. epidermidis* and *S. faecalis* giving an inhibition zone diameter up to 18.0 mm, including a disc diameter of 9.0 mm and a MIC of 95 - 170 μg ml^{-1}. The same alkaloids were found to be less active against *B. subtilis*, *E. coli* and *P. aeruginosa* with a MIC value of 429 to 600 μg ml^{-1}. In conclusion, they pointed out that the alkaloids, with concessine as the major one, appear to be responsible for much of the antibacterial activity of the stem bark of *H. pubescens*.

From the present study, it is evident that the herbal products with antibacterial activity could be effectively used to control pathogenic microbes in grouper aquaculture practice.

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