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Identification and Antimicrobial Activity of Combined Extract from *Azadirachta indica* and *Ocimum sanctum*

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Abstract

The chemical constituents of decoction (individual) and concoction (mixed) of ethanolic leaf extracts from *Azadirachta indica* (neem) and *Ocimum sanctum* (tulsi) were analyzed by gas chromatography-mass spectrophotometery (GC-MS). Decoctions of *A. indica* and *O. sanctum* had 24 and 33 constituents, respectively. Mixed together, 26 compounds were identified. Four major (high percentage) compounds were identified in *A. indica*: n-hexadecanoic acid (14.34%), phytol (19.96%), 9,12,15-octadecatrienoic acid, (Z,Z,Z)- (18.57%), and vitamin E (11.37%). Three major compounds were identified in *O. sanctum*: phenol,2-methoxy-3-(2-propenyl) (15.32%), 9,12,15-octadecatrienoic acid,(Z,Z,Z)- (16.94%), and 9,12,15-octadecatrienoic acid, methyl ester,(Z,Z,Z)- (22.05%). Three major compounds were identified in the mixed extract: n-hexadecanoic acid (16.58%), phenol,2-methoxy-3-(2-propenyl) (20.62%), and 9,12,15-octadecatrienoic acid,(Z,Z,Z) (25.98%). Four of the compounds in the mixed extract were new: eudesma-4(14),11-diene (0.18%), 6-azabicyclo[3.2.1]octane (0.51%), cyclohexane,1-ethyl-1-methyl-2,4-bis(1-methylenyl)-,[1S-(1α,2β,4β)]-β-Elemen (0.77%), and globulol (1.45%). The mixed extract had a high level of antimicrobial activity against fish pathogens as indicated by zone of inhibition, minimum inhibitory concentration, and minimum bactericidal concentration.

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Introduction

Aeromonas hydrophila, a heterotrophic gram-negative pathogenic bacterium prevalent in marine and freshwater ecosystems, causes motile Aeromonas septicemia (MAS) and associated economic losses in fisheries worldwide. It is responsible for a range of diseases in fish and amphibians including hemorrhagic septicemia (Karunasagar et al., 1995; Leung et al., 1995), where lesions lead to scale shedding, hemorrhages in the gills and anal area, ulcers, exophthalmia, and abdominal swelling. Other pathogens have been identified in aquatic systems, causing a wide range of fish diseases, including *Staphylococcus aureus* (Nemetz and Shotts, 1992), *Staphylococcus epidermidis* (Gunn et al., 1982), *Pseudomonas aeruginosa* (Govan and Deretic, 1996), and *Vibrio harveyi* (Govindachari, 1992)

As a result, alternative antimicrobial agents have been sourced from natural products. Traditional health remedies are popular with about 80% of the world's population in Asia, Latin America, and Africa, with minimal reported side effects. Medicinal plants have been model sources of medicines as they are a reservoir of chemical agents with therapeutic properties (Bhuvaneswari and Balasundaram, 2006; Bai et al., 2009). Pharmaceutical companies have spent considerable resources in the development of new therapeutic products from plants.

Azadirachta indica is a tree from the mahogany family indigenous to India and southeast Asia. Commonly called the neem tree, it contains a vast amount of bioactive compounds that are chemically diverse and is an important alternative herbal therapy (Subapriya and Nagini, 2005). Neem extract suppresses several species of pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella typhosa*, and *Mycobacterium tuberculosis* (Chaurasia and Jain, 1978; Rao et al., 1986) and arrests the growth of *Salmonella paratyphi* and *Vibrio cholerae* (Rao et al., 1986).

Ocimum sanctum (tulsi), of the Lamiaceae (mint) family, grows in India and Suriname. Its leaf extract has been used to treat a variety of conditions including catarrhal bronchitis, dysentery, and skin diseases. Its herbal extract produces hypoglycemic effects in rats. Aqueous and solvent extracts of tulsi stimulate humoral and cellular immunity in rats (Platel and Srinivasan, 2000) and aids in ulcer healing, which involves a combination of wound retraction and re-epithelialization, and promotes anti-ulcer activity (Godhwani et al., 1988; Perini et al., 2003).

Given their antimicrobial abilities, the potentially active compounds of neem and tulsi should be identified. The rational exploitation of plant materials, both in traditional medicine and in the empirical development of new antibacterial drugs, provides a potentially productive avenue of research. In this study, we used GC-MS and antimicrobial activity against fish pathogens to identify principle and new compounds in individual and combined extracts of *A. indica* and *O. sanctum.*

Materials and Methods

Preparation of extracts. Fresh leaves of *A. indica* (neem) and *O. sanctum* (tulsi) were collected between April and June 2007 at Tiruchirapalli, Tamil Nadu, India. The leaves were surface sterilized with 0.1% mercuric chloride (w/v) solution, washed thoroughly in running tap water for 10 min, and shade dried for 10 days until the weight was constant at room temperature (32-35°C) for 5 days. Each sample was finely powdered in an electric blender. The ethanolic decoctions and concoction were obtained using the procedures of Iwalokun et al. (2001). Twenty grams, each, of *A. indica* and *O. sanctum* were extracted successively with 60 ml of ethanol using a Soxhlet apparatus for 48 h at 45°C for individual and combined (1:1) extracts. The ethanolic extracts were filtered thoroughly through Whatman filter paper (no. 1) and concentrated in a rotary vacuum evaporator at 50°C. The obtained residues were stored in a freezer at -80°C until GC-MS analysis and antimicrobial activity tests.

Gas chromatography-mass spectrometry analysis (GC-MS). One µl of decoction and concoction extracts were injected and analyzed by GC-MS. The chemical compositions of the ethanolic leaf extracts were determined by the Technological Development Center (PADETEC) of the Federal University of Ceara using GC-MS with a Hewlett-Packard 5971 GC/MS apparatus (Avondale, PA, USA) under the following conditions: a 0.25 mm × 30 m polydimethylsiloxane DB-1 fused silica capillary column with a film thickness of 0.10 µm; helium as the carrier gas (1 ml/min); injector temperature of 250ºC; detector temperature of 200ºC. The column temperature ranged 35-180ºC/min at 4ºC V/min, then 180-280ºC at 20ºC V/min. Mass spectra were obtained by electronic impact 70 eV. The compounds were identified by comparison of retention indices (RRI) with those reported in related literature and by comparison of their mass spectra with the Wiley library (Lin et al., 1999) or published mass spectra (Massada, 1976).

Bacterial strains. To assay antibacterial activity, ethanolic extracts were dissolved in 5% DMSO to a final concentration of 100 mg/ml. *In vitro* antimicrobial activities of individual and combined extracts were evaluated against six fish pathogens obtained from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India: *Aeromonas hydrophila* (MTCC 646), *Staphylococcus aureus* (MTCC 87), *Streptococcus epidermidis* (MTCC 3382), *Pseudomonas aeruginosa* (MTCC 1034), *Vibrio harveyi* (MTCC 7771), and *Vibrio vulnificus* (MTCC 1145). The tested organisms were grown on Mueller-Hinton agar (MHA, Hi-Media) and stored at -4°C; sub-cultures were grown and checked for purity every two weeks.

Susceptibility test. The inhibition zones of the extracts were determined according to guidelines of the National Committee for Clinical Laboratory Standards (Adams, 2001). The test bacterial strains were inoculated into MHA Hi-Media and incubated for 3-6 h at 35°C in a shaker until the culture turbidity changed to 0.5 on the McFarland unit. The final inoculum of each strain was adjusted to approximately 5 × 104 cfu/ml. Susceptibility tests followed a modified agar-well diffusion method (NCCLS, 1993). One ml of this

standard suspension, in each test bacterial strain, was spread evenly on MHA plates using an 'L' sterile glass rod spreader, after which the plates were allowed to dry at room temperature. Subsequently, 6-mm diameter wells in triplicate were bored in the agar and 100 µl from each extract (i.e., decoction or concoction) were reconstituted in 5% DMSO/distilled water to transfer into the wells. The plates were incubated at room temperature for 2 h to allow diffusion of the extract into the agar, then incubated at 37°C for 24 h. The zone of inhibition (diameter) was measured to the nearest millimeter. Tetracycline (25 µg/ml) was used as a positive control and 5% DMSO as a negative control. The tests were performed in duplicate for each microorganism.

Minimum inhibitory concentration (MIC). The MIC of the ethanolic extracts was determined by macro broth dilution assay (Adams, 2001). Two-fold serial dilution of the ethanolic decoctions and concoction (0.1-100 mg/ml), the negative control (5% DMSO), and the positive control (tetracycline; 0.125-512 µg/ml) were prepared in tubes with MHA. The dilutions were seeded with test organisms at the standard concentration of 5 × 104 cfu/ml. The tubes were incubated at 37°C for 24 h and then examined for growth. The zone of inhibition of the lowest extract or control concentration was taken as the MIC.

Minimal bactericidal concentration (MBC). MBC was determined by aspirating 0.1 ml of the culture medium from tubes (in the macro broth MIC assay for ethanolic extracts) showing no apparent growth and subculturing it on fresh MHA at 37°C for 24 h. MBC was read as the least concentration showing no visible growth on MHA subculture.

Results

Identification of compounds. The chemical constituents of the ethanolic decoction and concoction leaf extracts obtained from *A. indica* and *O. sanctum* are listed in order of their elution from the Elite-1 (100% dimethyl poly siloxane) 30 m x 0.25 mm ID x 1 l df capillary column. Using GC-MS, 24 chemical constituents were identified from the ethanolic leaf extract of *A. indica* (Table 1). Of these, four compounds constituted over 10% of the extract and are considered major constituents. Thirty-three constituents were identified from the ethanolic leaf extract of *O. sanctum*; three are considered major constituents (Table 2). Twenty-six constituents were identified from the concoction, with three major constituents above 10% (Table 3).

Antimicrobial activity. The ethanolic leaf extracts of *A. indica* and *A. indica+O.sanctum* showed stronger antibacterial activity against *A. hydrophila* than the *O. sanctum* extract (Table 4). The positive control, tetracycline, showed strong inhibition against *A. hydrophila* while the negative control, DMSO, showed no inhibitory action. The least antimicrobial activity was found against *V. vulnificus*. Antimicrobial activity against the tested strains was ranked in the following order: *A. hydrophila<S. aureus<V. harveyi<S. epidermidis<P. aeruginosa<V. vulnificus*. The MIC of the combined extract was lower than that of either decoction or tetracycline against *A. hydrophila*

and moderate against *S. aureus*. This trend was similar in the MBC activity, which were within two-fold dilutions of the MIC.

Table 1. Chemical constituents from ethanolic *Azardirachta indica* leaf extract using GC-MS.

	RT	*MF*	*MW*	*Peak (%)*	*Compound*
**1	4.10	$C_3H_8O_3$	92	2.57	Glycerin
2	4.85	$C_6H_{10}O_2$	114	0.90	2-Hexenoic acid,(E)-
3	5.40	$C_9H_{13}N$	135	0.81	Benzeneethanamine,α-methyl-
**4	6.14	$C_5H_6N_2O_2$	126	1.94	Thymine
**5	7.60	$C_6H_8O_4$	144	3.14	4H-Pyran-4-one,2,3-dihydro-,5-dihydroxy-6-methyl-
6	8.02	$C_{15}H_{19}NO_7$	325	0.41	Glucosamine,N-acetyl-N-benzoyl-
7	9.01	$C_6H_6O_3$	126	0.79	2-Furancarboxadehyde,5-(hydroxymethyl)-
**8	12.53	$C_{10}H_{12}O_2$	164	6.26	Eugenol
9	13.86	$C_{15}H_{24}$	204	0.40	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R-(2α,4aα,8aβ)]-
10	14.73	$C_{15}H_{24}$	204	0.49	Caryophyllene
11	14.83	$C_{15}H_{24}$	204	0.78	γ-Elemene
12	9.01	$C_6H_6O_3$	126	0.79	2-Furancarboxadehyde,5-(hydroxymethyl)-
**13	17.45	$C_{12}H_{24}O_2$	200	2.90	Dodecanoic acid
14	17.96	$C_{15}H_{24}$	204	0.83	Azulene,1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylenyl)-,[1R-(1α,3aβ,4α,7β)]-
15	18.45	$C_{15}H_{24}O$	220	0.91	Caryophyllene oxide
**16	18.97	$C_6H_{12}O_6$	180	4.28	d-Mannose
**17	21.22	$C_{15}H_{24}O$	220	1.56	Tricyclo[5.2.2.0(1,6)]undecan-3-ol,2-methylene-6,8,8-trimethyl-
**18	23.58	$C_{20}H_{40}O$	296	2.23	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
19	24.43	$C_{18}H_{32}O_2$	280	0.47	9,12-Octadecadienoic acid,(Z,Z)-
*20	25.96	$C_{16}H_{32}O_2$	256	14.34	n-Hexadecanoic acid
*21	28.79	$C_{20}H_{40}O$	296	19.96	Phytol
*22	29.27	$C_{18}H_{30}O_2$	278	18.57	9,12,15-Octadecatrienoic acid,(Z,Z,Z)-
**23	29.61	$C_{18}H_{36}O_2$	284	3.76	Octadecanoic acid
*24	37.15	$C_{29}H_{50}O_2$	430	11.37	Vitamin E

* Major constituent (≥10%), ** Minor constituent (1-10%)

Table 2. Chemical constituents from ethanolic *Ocimum sanctum* leaf extract using GC-MS.

	RT	*MF*	*MW*	*Peak (%)*	*Compound*
**1	4.50	$C_6H_{14}O_5$	166	1.74	Diglycerol
2	5.40	$C_9H_{13}N$	135	0.07	Benzeneethanamine,α-methyl-
3	6.27	$C_5H_6N_2\ O_2$	126	0.23	Thymine
4	6.65	$C_9H_{17}N$	69	0.55	1H-Pyrrole,2,5-dihydro-
5	7.64	$C_6H_8O_4$	144	0.45	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-
6	8.18	$C_{15}H_{19}No_7$	325	0.11	Glucosamine,N-acetyl-N-benzoyl-
**7	9.16	$C_6H_6O_3$	126	1.23	2-Furancarboxadehyde,5-(hydroxymethyl)-
8	9.28	C_8H_8O	120	0.20	Benzofuran,2,3-dihydro-
9	11.54	$C_{10}H_{10}O_2$	150	0.08	2-Methoxy,4-vinylphenol
*10	12.73	$C_{10}H_{12}O_2$	164	15.32	Phenol,2-methoxy-3-(2-propenyl)-
11	13.39	$C_8H_8O_3$	152	0.69	Vanillin
**12	14.78	$C_{15}H_{24}$	204	1.00	Caryophyllene
13	15.98	$C_{15}H_{24}$	204	0.27	a-Guaiene
14	16.54	$C_{15}H_{24}$	204	0.67	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R-(2α,4aa,8aa)]-
15	17.53	$C_{16}H_{26}O_2$	250	0.16	Formic acid,3,7,11-trimethyl-1,6,10-dodecatrien-3-yl ester
**16	18.54	$C_{15}H_{24}O$	220	1.48	Caryophyllene oxide
**17	20.10	$C_{15}H_{26}O$	222	1.05	Epiglobulol
18	20.97	$C_{10}H_{12}O_4$	196	0.14	Benzeneacetic acid,4-hydroxy-3-methoxy-,methyl ester
19	21.20	$C_{15}H_{24}O$	220	0.62	Ledene oxide-(II)
20	21.56	$C_{15}H_{24}O$	220	0.67	Isoaromadendrene epoxide
21	23.37	$C_{28}H_{48}O$	400	0.26	Cholestan-3-ol,2-methylene-,(3a,5a)
**22	23.73	$C_{20}H_{40}O$	296	1.75	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
*23	25.61	$C_{18}H_{30}O_2$	278	16.94	9,12,15-Octadecatrienoic acid,(Z,Z,Z)-
**24	26.46	$C_{16}H_{32}O_2$	256	9.45	n-Hexadecanoic acid
25	29.07	$C_{20}H_{40}O$	296	0.87	Phytol
*26	30.06	$C_{19}H_{32}O_2$	292	22.05	9,12,15-Octadecatrienoic acid,methyl ester, (Z,Z,Z)-
**27	30.25	$C_{18}H_{36}O_2$	284	3.59	Octadecatrienoic acid
**28	34.43	$C_{10}H_{12}O$	148	3.16	Estragole
29	35.51	$C_{27}H_{43}CIO$	418	0.46	4-Chlorocholest-4-en-3-one
**30	35.74	$C_{24}H_{34}O_4$	386	2.36	Pregn-5-en-20-one,3-(acetyloxy)-16,17-epoxy-6-methyl-,(3a,16a)-
31	36.58	$C_{27}H_{43}CIO$	418	0.72	4-Chlorocholest-4-en-3-one
**32	42.06	$C_{30}H_{50}$	410	6.94	2,6,10,14,18,22-Tetracosahexaene 2,6,10,15,19,23-hexamethyl-,(all-E)-[All trans Squalene]
**33	42.61	$C_{30}H_{50}$	410	4.73	Squalene

* Major constituent (≥10%); ** Minor constituent (1-10%)

Table 3. Chemical constituents from mixed ethanolic *Azardirachta indica* and *Ocimum sanctum* leaf extract using GC-MS.

	RT	*MF*	*MW*	*Peak (%)*	*Compound*
**1	4.24	$C_3H_8O_3$	92	1.38	Glycerin
2	4.84	$C_6H_{10}O_2$	114	0.11	2-Hexenoic acid,(E)-
3	5.38	$C_9H_{13}N$	135	0.15	Benzeneethanamine,α-methyl-
4	6.17	$C_5H_6N_2O_2$	126	0.60	Thymine
5	6.61	$C_7H_{13}N$	111	0.51	6-Azabicyclo[3.2.1]octane
6	7.58	$C_6H_8O_4$	144	0.72	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-
7	8.03	$C_{15}H_{19}No_7$	325	0.13	Glucosamine,N-acetyl-N-benzoyl-
**8	9.05	$C_6H_6O_3$	126	1.59	2-Furancarboxadehyde,5-(hydroxymethyl)-
*9	12.66	$C_{10}H_{12}O_2$	164	20.62	Phenol,2-methoxy-3-(2-propenyl)-
10	13.33	$C_8H_8O_3$	152	0.80	Vanillin
11	13.87	$C_{15}H_{24}$	204	0.77	Cyclohexane,1-ethyl-1-methyl-2,4-bis(1-methylenyl)-,[1S-(1α,2β,4β)]-β-Elemen
**12	14.75	$C_{15}H_{24}$	204	1.56	Caryophyllene
13	16.31	$C_{15}H_{24}$	204	0.18	Eudesma-4(14),11-diene
14	16.52	$C_{15}H_{24}$	204	0.80	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R-(2α,4aα,8aβ)]-
**15	18.51	$C_{15}H_{24}$	204	2.14	γ-Elemene
16	20.07	$C_{15}H_{26}O$	222	1.45	Globulol
17	21.18	$C_{15}H_{24}O$	220	0.41	Ledene oxide-(III)
18	21.53	$C_{15}H_{24}O$	220	0.81	Isoaromadendrene epoxide
**19	23.75	$C_{20}H_{40}O$	296	2.45	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
*20	26.45	$C_{16}H_{32}O_2$	256	16.58	n-Hexadecanoic acid
**21	29.16	$C_{20}H_{40}O$	296	5.14	Phytol
*22	30.10	$C_{18}H_{30}O_2$	278	25.98	9,12,15-Octadecatrienoic acid,(Z,Z,Z)
**23	30.30	$C_{18}H_{36}O_2$	284	2.57	Octadecatrienoic acid
**24	35.94	$C_{24}H_{34}O_4$	386	3.09	Pregn-5-en-20-one,3-(acethyloxy)-16,17-epoxy-6-methyl,(3β,16α)-
**25	36.88	$C_{27}H_{43}CIO$	418	1.55	4-Chlorocholest-4-en-3-one
**26	43.17	$C_{30}H_{50}$	410	7.19	Squalene

* Major constituent (≥10%), ** Minor constituent (1-10%)

Table 4. Zone of inhibition, minimum inhibitory concentration, and minimum bactericidal concentration of tetracycline, ethanolic decoctions, and a concoction of *Azadirachta indica* and *Ocimum sanctum*.

	Tetracycline (µg/ml)	*Azdirachta indica (mg/ml)*	*Ocimum sanctum (mg/ml)*	*A. indica+O. sanctum (mg/ml)*
Zone of inhibition (mm)				
Aeromonas hydrophila	25	20*	15*	23
Staphylococcus aureus	23	18*	12*	20
Streptococcus epidermidis	20	11*	9*	13*
Pseudomonas aeruginosa	24	13*	11*	15*
Vibrio harveyi	18	17	12*	19
Vibrio vulnificus	15	5	-	7*
Minimum inhibition concentration (MIC)				
Aeromonas hydrophila	5	3.68	3.82	3.20
Staphylococcus aureus	6	4.20	4.28	3.46
Streptococcus epidermidis	6	-	-	4.20
Pseudomonas aeruginosa	8	-	-	4.82
Vibrio harveyi	7	5.40	4.46	3.75
Vibrio vulnificus	6	6.74	5.80	4.46
Minimum bactericidal concentration (MBC)				
Aeromonas hydrophila	18	12.88	14.34	12.62
Staphylococcus aureus	22	18.82	18.74	14.20
Streptococcus epidermidis	30	-	-	-
Pseudomonas aeruginosa	26	-	-	18.20
Vibrio harveyi	24	22.30	16.72	16.70
Vibrio vulnificus	34	-	-	-

* Significantly different from the positive control (tetracycline) at $p<0.05$.

Discussion

We identified 24 and 33 compounds from the individual ethanolic leaf extracts of neem and tulsi, respectively. More than 140 chemical compounds have been isolated from different parts of neem (Subapriya and Nagini, 2005) and the chemistry and structure of approximately 135 have been reviewed (Biswas et al., 2002). They include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives gedunin and vilasinin, and non-isoprenoids such as several proteins, carbohydrates, sulphurous compounds, and flavonoids. In our study, four new compounds were identified: 6-azabicyclo[3.2.1]octane (0.51%), cyclohexane,1-ethyl-1-methyl-2,4-bis(1-methylenyl)-,[1S-(1α,2β,4β)]-β-Elemen (0.77%), eudesma-4(14), 11-diene (0.18%), and globulol (1.45%). These four compounds were analyzed by a drug-likeness activity test using online software. Drug effectiveness was very high in the new compounds compared to other constituents (data not shown). High antimicrobial activity was reported earlier in the same concoction extract (Harikrishnan and Balasundaram, 2005).

The ethanolic leaf extracts of *A. indica* and *A. indica+O. sanctum* had stronger antibacterial activity against *A. hydrophila* than *O. sanctum*. Similarly, the MIC and MBC of the combined extract against *A. hydrophila* was lower than in extracts of either *A. indica* or *O. sanctum*. In treatments using the combinations *Rhizoma coptidis+Radix scutellariae*, *Galla chinensis+Radix et Rhizoma rhei*, *G. chinensis+R. scutellariae*, and *R. et Rhizoma rhei+R. scutellariae*, the MIC was significantly lower (≤4 mg/ml) than in treatments using *R. coptidis*, *R. et Rhizoma rhei*, or *Flos lonicerae* (≥32 mg/ml), alone (Bai et al., 2009). Although the mechanisms by which microorganisms generally survive the action of antimicrobial agents are poorly understood, the combined *A. indica+O. sanctum* extract may affect the cell walls of organisms.

Moderate MIC activity was noted in both decoctions and the concoction. The highest MIC activity was noted in *A. hydrophila* and *S. aureus*. A similar trend was noted in MBC activity. The bacterial strains presented MBC that were within two-fold dilutions of the MIC. Other reports show similar or higher MIC (Ramanoelina et al., 1987; Janssen et al., 1989). The highest MIC was no activity against *S. epidermidis* and *P. aeruginosa.*

Our results agree with previous findings that natural products yield a variety of chemical components (Seger et al., 2004; Braga et al., 2005; Michielin et al., 2005). Given this, it is no surprise that 7000 pharmaceutically important compounds have been derived from plants and are sold to treat disease. The treatment of bacterial fish pathogens is problematic because high concentrations of antibiotics or treatment chemicals can produce antibiotic-resistant strains, failures, or relapses, and side effects. In contrast, the application of high concentrations of individual or combined herbal extracts produce no resistant strains or side effects.

The use of antibiotics, such as tetracycline, may result in residues, and cause the emergence of drug-resistant bacterial strains, which may be transferable to humans (Shao, 2001). Some antimicrobial and chemical agents used to treat fish diseases are increasingly ineffective. There is growing concern over the increase in multi-drug-resistant bacteria in human and veterinary medicine (Bryan, 1984). Natural products from plants and microorganisms contain many compounds. Researchers have been studying traditional folk medicines to discover the scientific basis of their remedial effects and seek new compounds for development of novel therapeutic agents (Cragg et al., 1997).

This is of great significance, especially in Asia where the cost of obtaining medical care is high and alternatives to antibiotics and chemotherapy are cost-effective. Our results were confirmed by *in vitro* antimicrobial activity assay, where the combined extract showed better antimicrobial activity than decoction extracts. Purification and characterization of the active components may reveal new agents important to treatment of fish diseases.

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References

Adams R.P., 2001. *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy*. Allured, Carol Stream, IL.

Bai D.Q., Li R., Xing K.Z., Guo Y.J., Chen C.X., Qiao X.T., Mao H.T. and G.X. Zhu, 2009**.** *In vitro* antibacterial activity of herbal medicines and combinations of herbal medicines and antibiotics against *Edwardsiella tarda*. *Isr. J. Aquac. - Bamidgeh,* 61:27-34.

Biswas K., Chattopadhyay I., Banerjee R.K. and U. Bandyopadhyay, 2002. Biological activities and medicinal properties of neem (*Azadirachta indica*). *Curr. Sci*., 82:1336-1345.

Braga M.E.M., Ehlertb P.A.D., Ming L.C. and A.A. Meireles, 2005. Supercritical fluid extraction from *Lippia alba*: global yields, kinetic data, and extract chemical composition. *J. Supercritical Fluids*, 34:149-156.

Bryan L.E., 1984. *Antimicrobial Drug Resistance*. Academic Press, New York. pp. 255-256.

Bhuvaneswari R. and C. **Balasundaram**, 2006. Traditional Indian herbal extracts used *in vitro* against growth of the pathogenic bacteria - *Aeromonas hydrophila*. *Isr. J. Aquac. - Bamidgeh,* 58:89-96*.*

Chaurasia S.C. and P.C. Jain, 1978. Antibacterial activity of essential oils of four medicinal plants. *Indian J. Hosp. Pharm*.**,** 28:166-168.

Cragg G.M., Newman D.J. and K.M. Snader, 1997. Natural products in drug discovery and development. *J. Nat. Prod*., 60:52-60.

Godhwani S., Godhwani J.L. and D.S. Vyas, 1988. *Ocimum sanctum* - a preliminary study evaluating its immunoregulatory profile in albino rats. *J. Ethnopharmacol*., 24:193-198.

Govan J.R.W. and V. Deretic, 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev.*, 60:539-574.

Govindachari T.R., 1992. Chemical and biological investigations on *Azadirachta indica* (the neem tree). *Curr. Sci*., 63:117-122.

Gunn B.A., Singleton F.L., Peele E.R. and R.R. Colwell, 1982. A note on the isolation and enumeration of gram positive cocci from marine and estuarine waters. *J. Appl. Bacteriol*., 53:127-129.

Harikrishnan R. and C. Balasundaram, 2005. Antimicrobial activity of medicinal herbs *in vitro* against fish pathogen, *Aeromonas hydrophila. Fish Pathol*., 40:187-189.

Iwalokun B.A., Gbenle G.O., Adewole T.A. and K.A. Akinsinde, 2001. Shigellocidal properties of three Nigerian medicinal plants: *Ocimum*

gratissimum, *Terminalia avicennoides*, and *Momordica balsamina*. *J. Health Pop. Nutr.,* 19:331-335.

Janssen A.M., Scheffer J.J., Ntezurubanza L. and A. Baerheim Svendsen, 1989. Antimicrobial activities of some *Ocimum* species grown in Rwanda. *J. Ethnopharmacol*., 26:57-63.

Karunasagar I., Sugumar G. and I. Karrunasagar, 1995. Virulence characters of *Aeromonas* sp. isolated from EUS-affected fish. pp. 307-314. In: M. Sharif, J.R. Arthur, R.P. Subasinghe (eds.). *Diseases in Asian Aquaculture II*. Asian Fish. Soc., Manila.

Leung, K.Y., Low K.W., Lam T.J. and Y.M. Sin, 1995. Interaction of the fish pathogen *Aeromonas hydrophila* with tilapia, *Oreochromis aureus* (Steindachner), phagocytes. *J. Fish. Dis*., 18:435-447.

Lin J., Opoku A.R., Geheeb-Keller A.K., Hutchings A.D., Terblanche S.E., Jager A.K. and J. Van Staden, 1999. Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities. *J. Ethanophama*., 68:267-274.

Massada Y., 1976. *Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry*. Wiley, New York.

Michielin E.M.Z., Bresciani L.F.V., Danielski L., Yunes R.A. and S.R.S. Ferreira, 2005. Composition profile of horsetail (*Equisetum giganteum* L.) oleoresin: comparing SFE and organic solvents extraction. *J. Supercritical Fluids*, 33:131-138.

NCCLS, 1993. *Methods for Dilution in Antimicrobial Susceptibility Test. Approved Standard. M2-M5*. National Committee for Clinical Laboratory Standards, Villanova, PA.

Nemetz T.G. and E.B. Shotts, 1992. Zoonotic diseases. pp. 214–220. In: M.K. Stoskopf (ed.). *Fish Medicine*. W.B. Saunders, Philadelphia.

Perini R.F., Ma L. and J.L. Wallace, 2003. Mucosal repair and COX-2 inhibition. *Curr. Pharma. Design*, 9:2207-2211.

Platel K. and K. Srinivasan, 2000. Influence of dietary spices or their active principles on pancreatic digestive enzymes in albino rats. *Nahrung*, 44:42-46.

Ramanoelina A.R., Terrom G.P., Bianchini J.P. and P. Coulanges, 1987. Antibacterial action of essential oils extracted from Madagascar plants. *Arch. Inst. Pasteur Madagascar*, 53:217-226.

Rao D.V.K., Singh I., Chopra P., Chhabra P.C. and G. Ramanujalu, 1986. *In vitro* antibacterial activity of neem oil. *Indian J. Med. Res*., 84:314-316.

Seger C., Rompp H., Sturm S., Haslinger E., Schmidt P.C. and F. Hadacek, 2004. Characterization of supercritical fluid extracts of St. John's wort (*Hypericum perforatum* L.) by HPLC-MS and GC-MS. *Eur. J. Pharm. Sci*., 21:453-463.

Shao Z.J., 2001. Aquaculture pharmaceuticals and biologicals: current perspectives and future possibilities. *Adv. Drug Del. Rev*., 50:231-236.

Subapriya R. and S. Nagini, 2005. Medicinal properties of neem leaves: A review. *Curr. Med. Chem. Anticancer Agents*, 5:149-56.