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Combination Studies of *Oreganum Vulgare* Extract Fractions and Volatile Oil along with Ciprofloxacin and Fluconazole against Common Fish Pathogens

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ABSTRACT

Purpose: The study is aimed at finding new antibiotic therapy for aquaculture due to potential of bacteria to develop resistance to the existing therapies. Use of large quantities of synthetic antibiotics in aquaculture thus has the potential to be detrimental to fish health, to the environment and wildlife and to human health. ***Methods:*** Antimicrobial potential of volatile oil and fractions of chloroform extract of *Oreganum vulgare* was evaluated alone and in the presence of standard antimicrobials against common fish pathogens by disc-diffusion, agar well assay and two fold microdilution method by nanodrop spectrophotometric method. ***Results:*** The best results were represented by volatile oil followed by phenolic fraction by disc-diffusion, agar well and microdilution assays (Minimum inhibitory concentration). By the interaction studies, it was observed that the volatile oil and phenolic fraction were able to inhibit the pathogens at very low concentration compared to standard drugs. The fractional inhibitory concentration index (FICI) was calculated and volatile oil and phenolic fractions were found to be synergistic against *Pseudomonas fluorescens* and *Candida albicans*. ***Conclusion:*** The experimental data suggests the use of volatile oil and phenolic fraction in combination with standard antimicrobials to maintain healthy aquaculture with lesser adverse effects as compared to synthetic antibiotic therapy.

Introduction

Seafood has always been important for human being since time immortal as it is nutrient- rich and plays an important role in reducing health risks like cardiovascular complications. Consumption of seafood provides many benefits like neurological development during gestation and infancy.[1-4] Along with benefits, seafood may prove harmful if it is contaminated with pathogens, heavy metals and marine toxins. Bacteria are a major group of pathogens, which infects fishes all over world.[5] The infection may be transmitted to humans by exposure to infected organisms by any means. Major group of bacteria associated with pathogenicity of fish are Aeromonads, Pesudomonads and Edwardsiella tarda.[6,7] *Pseudomonas fluorescens* causes Red Skin Disease in fish.[8] *Pseudomonas aeruginosa* are opportunistic human pathogens that are one of the main causes of human infections. *Aeromonas* infection in human being causes several gastrointestinal syndromes like bloody mucoid stools, vomiting, abdominal pain and acute and self-limiting diarrhea. Not only bacteria, but also fungi are infectious agents in marine environment. *Candida albicans* is one of pathogenic fungi affecting killer whales. Control of fish disease is not only necessary for management of aquaculture but also for welfare of human being which can be possible due to chemotherapy.[9] Various marketed formulations available to control fish disease are chemicals like Malachite green, piperazine, formalin, copper sulfate, organic compounds like napthaquinones, tea tree oil and antibiotics naladixic acid, ciprofloxacin, triple sulfa etc. Although there is wide range of synthetic and semi-synthetic antibiotics and antifungal compounds present in market, none of them is completely effective and there is emergence of resistance. Moreover, if organic compounds obtained from natural sources exhibiting antimicrobial activities are given along with synthetic antibiotics, not only antibiotic resistance in humans but also in fishes can also be solved to a great extent.

The main objective of this study was to determine the interaction effect studies of isolated phenolic and non-phenolic fractions of chloroform extract of *Oreganum vulgare* Linn. (Lamiaceae) against common fish pathogenic strains *Pseudomonas fluorescens*, *Aeromonas hydophila*, *Candida albicans* along with ciprofloxacin and fluconazole for bacterial and fungal

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strains respectively. Antimicrobial interaction studies were performed by using disc diffusion method, agar well assay, minimum inhibitory concentration (MIC) by microdilution method, fractional inhibitory index (FICI) determination.

Materials and Methods

Plant Material and preparation of extracts

The freeze dried leaves of *Oreganum vulgare* Linn. were procured from Aum Agreefresh pvt. Ltd., Vadodara, Gujarat and were identified by the same company. The voucher specimen (Pcog 1101) was deposited in Department of Pharmaceutical sciences, Guru Jambheshwar University of Science and Technology for future references.

Crude drug (500 g) was placed in a closed flask with chloroform and after 24 h, filtered and concentrated in rotary vacuum to yield 12.5 g of paste like extract.[10] In order to separate the phenolic from non-phenolic fraction of the chloroform extract, a liquid-liquid extraction was done. In a seperating funnel, 2 g of the extract was diluted in 40 ml of chloroform and washed three times with 120 ml of 0.1 N sodium hydroxide. The chloroform phase was separated and was concentrated to obtain the crude non- phenolic fraction. To further purify this fraction, 0.3 g of it were diluted in ethanol and centrifuged at 3600×g at 10°C for 15 min. Ethanol was concentrated from the supernatant to obtain purified non- phenolic fraction. The basic aqueous phase was acidified with 6N HCl to pH 3.0 and 40 ml of chloroform was added to extract the phenolic fraction. The phenolic fraction was dissolved in chloroform and separated by preparative thin layer chromatography (TLC) on silica gel-G eluting with benzene- methanol 95:5.[10]

Extraction of volatile oil

Volatile oil was extracted from freeze dried leaves (1000 g) by hydro-distillation method by using clevenger's apparatus. The yellowish oil (16.6 ml, yield= 1.66 % v/w) obtained was separated from aqueous phase and dried over anhydrous sodium sulphate and stored at 4°C until used.

GC-MS analysis of Volatile oil

The oil sample was diluted with hexane in ratio of 1:100 and used for the further analysis. The quantitative analysis was done with the help of chromatographer in gas phase (Agilent 7890A GC system) equipped with MS detector (5975C inert XL EI/CI MSD), HP-5MS capillary column (Agilent 19091S-433: 1548.52849 HP-5MS 5% Phenyl Methyl Silox) having dimensions 30 m x 250 µm x 0.25 µm. The column temperature was programmed from initial 80°C upto 300°C. The temperature of the injector was fixed to 270°C. The debit of gas (helium) vector was fixed to 1ml/min and split injection with split ratio 50:1. The volume of injected sample was 2 µL of diluted oil in hexane (10%). The components were identified based on comparison of their relative retention time and mass spectra with those of standards, W9N08.L library data of the GC-MS system and literature data.

Bacterial strains and antibiotics

The microorganisms used for antimicrobial studies of volatile oil and extract were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. The bacterial strains used were *P.fluorescens* MTCC 7200 and *A.hydrophila* and fungal strain used was *C.albicans* MTCC 854. *A.hydrophila* was procured from slant cultures of Department of Biotechnology, CDLU, Sirsa. The media used for the growth and maintenance of microorganisms were nutrient agar (NA), for bacteria, potato dextrose agar (PDA) for fungi (Hi-media). The organic solvents used for extraction and fractionation of plant metabolites were of analytical grade.

Antimicrobial Screening

Disc Diffusion Assay

Antimicrobial activity of volatile oil was investigated along with ciprofloxacin and fluconazole using the standard method of diffusion disc plates on agar taking ciprofloxacin and fluconazole as positive control for bacterial and fungal strains respectively and DMSO as negative control.[11,12] For interaction effect studies, 0.25 mg ml^{-1} ciprofloxacin in DMSO was mixed with volatile oil in 1:1 concentration for antibacterial activity determination and 1:1 combination of 0.25 mg ml^{-1} of fluconazole dissolved in DMSO for the determination of antifungal activity. In this method 60 µl of 24 hr. old culture of test organism was inoculated on the agar plates and spread on to the surface of the agar with the help of sterilized glass spreader. After five minutes of inoculation of test organism, sterile paper discs (5mm diameter) were placed in each agar plate disc dipped in volatile oil and solution of volatile oil along with ciprofloxacin and fluconazole respectively. The bacterial cultures were incubated at 37°C for 18-24 h and fungal cultures at room temperature for 48 h. Zones of inhibition were measured. All the tests were done in triplicate.

Agar well assay

The preliminary investigation of the antibacterial activity of phenolic and non-phenolic fractions as well as interaction effect studies with that of synthetic antibiotics, ciprofloxacin and fluconazole was performed for bacterial and fungal strains respectively.[13] In this method 60 µl of 24 h old culture of test organism was inoculated on the agar plates and spread on to the surface of the agar with the help of sterilized glass spreader. After five minutes of inoculation of test organism, wells (2.5 mm diameter) were prepared with the help of sterilized steel cork borer.

Two wells of each plate were loaded with 60 µl of phenolic and non-phenolic fractions respectively. One well loaded well was loaded with 60 µl standard antibiotics viz. ciprofloxacin for bacterial strains and fluconazole for fungal strains were used as positive controls. One well was loaded with DMSO was used as a negative control. The bacterial cultures were incubated at 37°C for 18-24 h and fungal culture at room temperature for 48 h. For interaction effect studies, phenolic and non-phenolic fractions were mixed with 0.25 mg ml^{-1} of ciprofloxacin dissolved in DMSO in 1:1 combination and fluconazole dissolved in DMSO in 1:1 combination for bacterial and fungal strains respectively. Zones of inhibition were measured. Antimicrobial activity was determined by measuring zone of inhibition and compared with the growth inhibition results, obtained from standard microbial. The diameter (in mm) of zone inhibition was measured at cross-angles and the mean of two reading was taken. All the tests were done in triplicate.

Minimum Inhibitory Concentration (MIC) determination and comparison of MIC determination by spectrophotometric and visual methods and growth curve

MIC was determined by modified microdilution method.[14,15] The concentration of stock solutions of phenolic and non-phenolic fractions were 10 mg ml^{-1} and that of ciprofloxacin and fluconazole were 0.25 mg ml^{-1} in DMSO respectively for bacterial and fungal strains. Phenolic and non- phenolic fractions and volatile oil (0.5 ml each) were mixed with 0.5 ml of ciprofloxacin respectively. MIC of phenolic, non-phenolic fraction, volatile oil and ciprofloxacin was determined using two fold serial dilution method. For determination of interaction effect of phenolic, non-phenolic fractions and volatile oil, 0.5 ml of respective test sample were mixed with 0.5 ml of ciprofloxacin stock solution and 0.5 ml of fluconazole for bacterial and fungal strains respectively. MIC was determined using two fold serial dilution method. Tubes containing only bacterial suspensions and nutrient broth were used as positive control and negative control were the tubes with only nutrient broth.

Optical Densities (ODs) were measured for at 35°C using Thermo Scientific 2000/2000 C nanodrop spectrophotometer at 405 nm. OD of each replicate at before incubation (T_0) was subtracted from OD after incubation at 37° C (T_{24}) for bacterial cultures and at room temperature for fungal strains respectively. The adjusted OD of each control tube was then assigned a value of 100% growth. The percent inhibition of growth was thus determined using the formula:

Percent Inhibition = 1- (OD of tube containing test solution/OD of corresponding control tube) × 100.

The MIC is reported as the lowest concentration of test material which results in 100% inhibition of growth of the test organism. Visual MIC was determined by noting down the concentration of that first tube in which there is no appearance of turbidity after incubation of 24 h and it was compared with that of MIC determined by spectrophotometric method.

Fractional Inhibitory Concentration (FIC) Index Determination

The FIC index (FICI) was calculated by dividing the MIC of the combination of phenolic fraction, non-phenolic fraction, volatile oil and reference antibiotic respectively.[16]

FIC of vol. oil= MIC of vol. oil in combination with antibiotic drug/MIC of vol. oil

FIC of Phenolic Fraction= MIC of Phenolic Fraction in combination with antibiotic drug/ MIC of Phenolic Fraction

FIC of Non-Phenolic Fraction= MIC of Non-Phenolic Fraction in combination with antibiotic drug/ MIC of Non-Phenolic Fraction

FIC of antibiotic drug= MIC of antibiotic drug with particular fraction/MIC of drug

FICI (Vol. Oil) = FIC of Vol. oil+ FIC of antibiotic drug

FICI (Phenolic Fraction) = FIC of Phenolic Fraction+ FIC of antibiotic drug

FICI (Non-Phenolic Fraction) = FIC of Non-Phenolic Fraction+ FIC of antibiotic drug

Results and Discussion

GC-MS of volatile oil, Disc-diffusion and agar well assay

GC-MS analysis of volatile oil characterized and quantified total 35 compounds (Table 1). The major component of volatile oil *Oreganum vulgare* is carvacrol (86.5%), followed by p-cymene (7.2%), γ-Terpinene (0.6%), 3-Cyclohexen-1-ol (0.5%), δ-Cadinene (0.4%), β- Bisabolene (0.4%). The diameters of zones of inhibition of volatile oil, phenolic and non-phenolic fractions are shown in Table 2 against *P.fuorescens*, *A.hydrophila* and *C.albicans*. Non-phenolic fraction in terms of zone of inhibition was only effective against *A.hydrophila* while phenolic fraction and volatile oil showed antimicrobial activity against all the three tested strains.

MIC determination by spectrophotometric method and growth curve

Minimum inhibitory concentration is that concentration at which absorbance at time initiation time (T0) and after 24 h incubation, (T24) becomes equal. The MIC of non-phenolic fraction, phenolic fraction, volatile oil, combination of non-phenolic fraction, phenolic fraction and volatile oil respectively and ciprofloxacin in 1:1 ratio are shown in Table 2 and 3 were at 0.625, 0.01953, 0.00061, 0.156, 0.00122, 0.00030 mg ml^{-1} as compared to MIC of ciprofloxacin at 0.03900 mg ml^{-1} against *P.fluorescens* while against *A.hydrophila*, MIC of non-phenolic fraction, phenolic fraction, volatile oil, combination of non-phenolic fraction, phenolic fraction and volatile oil respectively and ciprofloxacin in 1:1

ratio was found to be 0.625, 0.156, 0.00030, 0.625, 0.00122, 0.00015 mg ml^{-1} as compared to MIC of ciprofloxacin at 0.00122 mg ml^{-1} (Table 3, 4). MIC exhibited by non-phenolic fraction, phenolic fraction, volatile oil, combination of non-phenolic fraction, phenolic fraction, volatile oil and fluconazole and fluconazole respectively was 5, 1.25, 0.01953, 0.07800, 0.00970, 0.00244 and 0.03900 mg ml^{-1} (Table 3, 4). A growth curve (% inhibition in concentration in mg ml^{-1}) was prepared for *P.fluorescens, A.hydrophila* and *C.albicans* in the presence of phenolic, non-phenolic fractions of chloroform extract and volatile oil respectively (Figure 1A, 1B and 1C respectively). The growth curve (Figure 1A) shows % inhibition of bacteria *P.fluorescens* at increasing concentrations (from 0.00015 mg ml^{-1} upto 10 mg ml^{-1}). The curve and Table 3 vividly indicates that at the concentration of 0.00015 mg ml^{-1} for non-phenolic fraction, combination of non-phenolic fraction and ciprofloxacin, phenolic fraction, combination of phenolic fraction and ciprofloxacin, volatile oil, combination of volatile oil and ciprofloxacin and ciprofloxacin, the % inhibition of *P.fluorescens* was found to be approximately 14%, 25%, 53%, 78%, 87% and 27% respectively. The % of inhibition of growth was found to be higher as the concentrations increased. The 100% inhibition of growth of bacteria was observed at 0.625, 0.156, 0.01953, 0.00122, 0.0006, 0.00030 and 0.03900 mg ml^{-1} for non-phenolic fraction, combination of non-phenolic fraction and ciprofloxacin, phenolic fraction, combination of phenolic fraction-ciprofloxacin, volatile oil, combination of volatile oil-ciprofloxacin and ciprofloxacin respectively. The curve indicates the superiority of phenolic fraction, combination of phenolic fraction and ciprofloxacin, volatile oil and volatile oil along with ciprofloxacin over ciprofloxacin alone against *P.fluorescens.* The growth curve (Figure 1B) and Table 3 depicts that at the lowest concentration chosen (0.00015mg ml^{-1}) non-phenolic fraction, non-phenolic fraction and ciprofloxacin, phenolic fraction, phenolic fraction in combination with ciprofloxacin, volatile oil, volatile oil and ciprofloxacin and ciprofloxacin the % inhibition of *A.hydrophila* was found to be 10, 25.7, 12.8, 78.5, 95.7, 100% respectively. The 100% inhibition concentration was 0.625, 0.625, 0.156, 0.00122, 0.00030 and 0.00015 mg/ml respectively. The curve indicates that volatile oil, volatile oil in combination with ciprofloxacin and phenolic fraction in combination with ciprofloxacin are more potent than ciprofloxacin against *A.hydrophila.* Figure 1C and Table 4 represents that at 0.00015 mg ml^{-1} concentration non-phenolic fraction showed least inhibition against *C.albicans* (2%) followed by phenolic fraction (8%), fluconazole (20%), non-phenolic fraction along with fluconazole (34%), phenolic fraction in combination with fluconazole (50%), volatile oil (48%) and volatile oil in combination with fluconazole (66.8%) respectively. Volatile oil, combination of volatile oil and fluconazole, phenolic fraction and fluconazole were found to have 100% inhibitory effect at less concentration (0.01953, 0.00244, 0.00970 mg ml^{-1} respectively) than that of fluconazole alone (0.03900 mg ml^{-1}).

Table 1. Gc-MS analysis of volatile oil of Oreganum vulgare Linn.

Chemical constituent	Retention Time	% of Chemical constituent
α-Thujene	3.482	0.076
α- Pinene	3.609	0.341
Camphene	3.851	0.114
1-Octen-3-ol	4.226	0.091
2-β- Pinene	4.300	0.060
3-Octanone	4.359	0.046
β- Myrcene	4.435	0.143
1-Phellandrene	4.756	0.029
α-Terpinene	4.989	0.333
Þ-cymene	5.153	7.249
Dl- limonene	5.240	0.179
1,8- Cineole	5.333	0.064
γ-Terpinene	5.883	0.642
Cis- Sabinenehydrate	6.116	0.058
α-Terpinolene	6.604	0.062
β-Linalool	6.840	0.154
3-Cyclohexen-1-ol	9.013	0.565
Benzenemethanol	9.2449	0.068
α-Terpineol	9.402	0.126
2-cyclohexen-1-one	11.039	0.053
m-Thymophenol	12.457	0.254
Carvacrol	12.857	86.586
Carvol	13.322	0.489
Eugenol	15.886	0.106
Caryophyllene	16.305	0.373
Aromadendrene	16.889	0.045
α- Humulene	17.332	0.063
Viridiflorene	18.582	0.037
β- Bisabolene	18.958	0.400
γ-Cadinene	19.142	0.152
δ- Cadinene	19.416	0.421
Caryophyllene oxide	21.186	0.271
Virdiflorol	22.820	0.094

It was reported that phenolic components of essential oil have the strongest antimicrobial activity, followed by aldehydes, ketones and alcohols.[17] In other research, the antimicrobial effect of volatile oil of *O. vulgare* on several Gram-positive and Gram-negative bacteria and saprophytic or foodborne pathogenic bacteria was investigated; results showed that this volatile oil has a strong antimicrobial activity. Polyphenols are well documented to have microbicide activities against a large number of pathogenic bacteria and fungal species.[18,19] The mechanisms responsible for phenolic toxicity to microorganisms include: adsorption and disruption of microbial membranes, interaction with enzymes and metal ion deprivation.[20,21]

Table 2. Measurement of zone of inhibition in mm of volatile oil, phenolic and non-phenolic fractions of chloroform extract of *Oreganum vulgare* against fish pathogenic strains.

Strain	NP	P	O	S
P.fuorescens	<10	12.34± 0.12	27.71± 1.31	32.60± 0.98
A.hydrophila	21.66± 1.31	22.28± 0.33	22.30± 1.11	26.57± 0.18
C.albicans	<10	<10	20.33± 0.64	16.43± 0.37

Note: The data are expressed as mean±error of mean (n=3). NP= non-phenolic fraction, P= phenolic fraction, O= volatile oil, S= standard drug (ciprofloxacin for bacterial strains and fluconazole for fungal strains).

Table 3. MIC and percent growth inhibition of volatile oil, phenolic, non-phenolic fractions of chloroform extract alone and in combination with ciprofloxacin against *P.fluorescens* and *A.hydrophila* by microdilution method.

	P.fluorescens							*A.hydrophila*						
Conc. (mg/ml)	**NP**	**NP+C**	**P**	**P+C**	**O**	**O+C**	**C**	**NP**	**NP+C**	**P**	**P+C**	**O**	**O+C**	**C**
0.00015	13.3	25	53	78	95	86.6	26.6	10	25.7	12.8	78.5	95.7	100	76.2
0.00030	23.3	33	58.3	88.3	96.6	100	33.3	22.8	34.2	27.1	85.7	100	>100	81.4
0.00061	30	42.3	63.3	92.3	100	>100	40	31.4	42.8	35.7	94.2	>100	>100	92.8
0.00122	40.6	55	66.7	100	>100	>100	53.3	40	51.4	44.2	100	>100	>100	100
0.00244	51.6	61.6	75	>100	>100	>100	61.6	50	52.8	54.2	>100	>100	>100	>100
0.00488	53.3	63.3	85	>100	>100	>100	73.3	61	64.2	67.1	>100	>100	>100	>100
0.00970	60	74.3	91.6	>100	>100	>100	80	62.8	65.7	77.1	>100	>100	>100	>100
0.01953	71.6	77	100	>100	>100	>100	93.3	71.4	72.8	85.1	>100	>100	>100	>100
0.03900	72.3	83.3	>100	>100	>100	>100	100	74.2	75.7	87.1	>100	>100	>100	>100
0.07800	81.6	93	>100	>100	>100	>100	>100	81.4	83.7	91.4	>100	>100	>100	>100
0.15600	87	100	>100	>100	>100	>100	>100	82.8	84.2	100	>100	>100	>100	>100
0.31200	91	>100	>100	>100	>100	>100	>100	90	92.8	>100	>100	>100	>100	>100
0.62500	100	>100	>100	>100	>100	>100	-	100	100	>100	>100	>100	>100	-
1.25000	>100	>100	>100	>100	-	-	-	>100	>100	>100	>100	-	-	-
2.50000	>100	>100	>100	>100	-	-	-	>100	>100	>100	>100	-	-	-
5.00000	>100	>100	>100	>100	-	-	-	>100	>100	>100	>100	-	-	-
10.0000	>100	-	>100	-	-	-	-	>100	-	>100	-	-	-	-

NP= non-phenolic fraction, P= phenolic fraction, O= volatile oil, S= standard drug ciprofloxacin, % in= inhibition, MIC= minimum inhibitory concentration, - = Dilution was not made in this concentration range for the given sample.

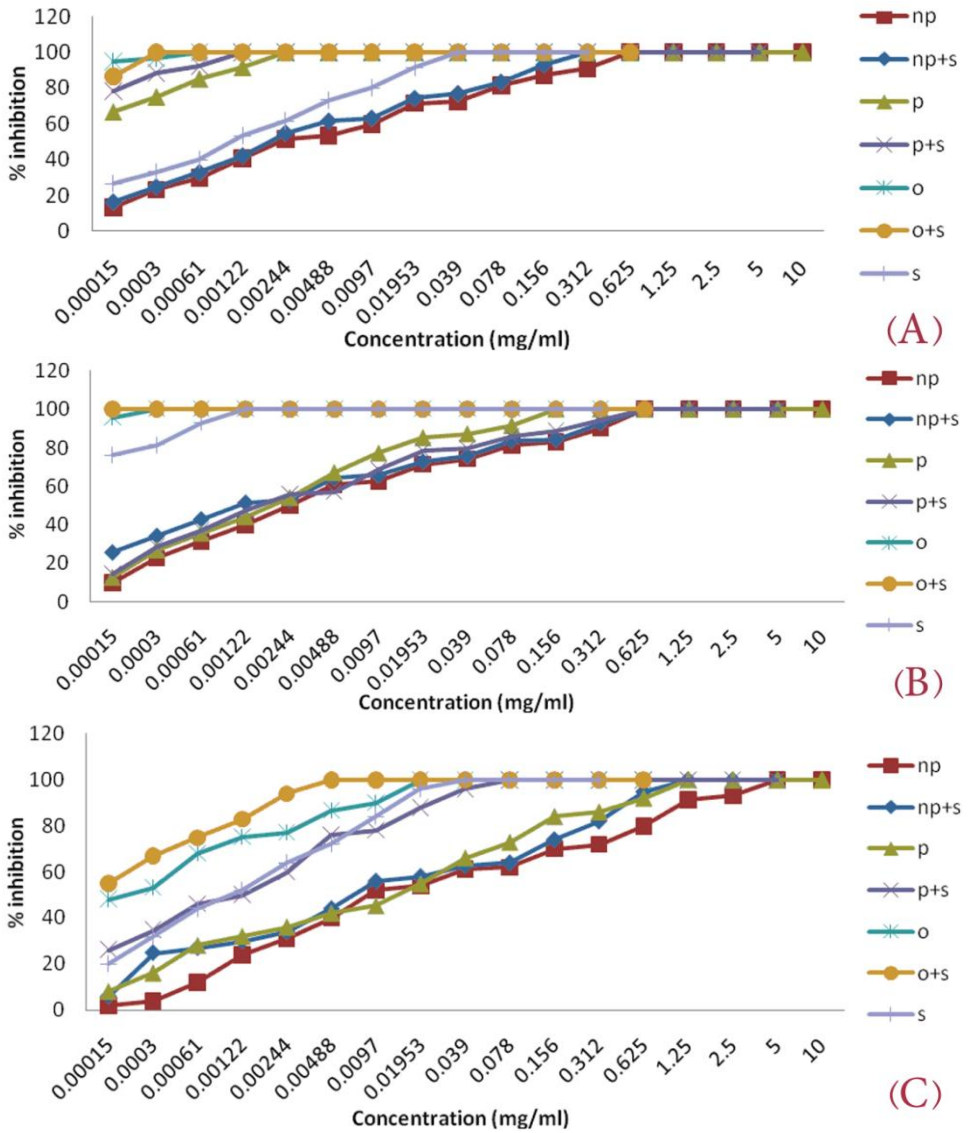

Figure 1. Growth curve (% inhibition against concentration in mg/ml) of (A) P.fluorescens (B) A.hydrophila (C) C.albicans in presence of phenolic, non-phenolic fractions and volatile oil alone and in combination with ciprofloxacin and fluconazole.

Fractional Inhibitory Concentration Index

Fractional inhibitory concentration was calculated to calculate FIC index which is an indicator of degree of interaction between standard drugs ciprofloxacin and fluconazole along with volatile oil, phenolic and non-phenolic fractions respectively for bacterial and fungal strains. FIC for volatile oil was found to be 0.491, 0.500 and 0.124 and that of phenolic fraction was found to be 0.062, 0.007 and 0.007 respectively against *P.fluorescens, A.hydrophila* and *C.albicans* (Table 5). Synergy is defined as an FIC index of ≤0.5. Indifference was defined as an FIC index of ≥0.5 but of ≤4.0. Antagonism was defined as an FIC index of >4.0.[22] Synergism was shown by volatile oil with a FICI of 0.498 and phenolic fraction with FICI of 0.093 respectively against *P.fluorescens* and 0.186 and 0.255 respectively against *C.albicans*. Indifference was exhibited by volatile oil phenolic fraction with FICI of 0.622 and 1.007 respectively against *A.hydrophila* and by non-phenolic fraction with FICI of 2.015 against *C.albicans.* Antagoistic interaction effect was observed for non-phenolic fraction with FICI of 4.249 and 513.295 against *P.fluorescence* and *A.hydrophila* (Table 5). The literature studies reveal that essential oil of *Oreganum vulgare* is effective against various strains of *Candida albicans* (13 to 15 mm diameter of zone of inhibition) and synergism is exhibited along with amphotericin B (16 to 23 mm diameter of zone of inhibition) and along with nystatin (17 to 20.1 mm) shown by disc-diffusion assay.[23,24] Very scanty information is available on this plant for the interaction studies with synthetic antibiotics as well as with other natural products. Synergism was shown by *Oreganum vulgare* and *Rosmarinus officinalis* combination against *S.aureus, L.monocytogens, Aeromonas hydrophila, Yersinia enterocolitica, P.fluorescens*.[25] Extracts of *Origanum vulgare* and *Vaccinium macrocarpon* presented a combined antimicrobial effect potentialized against *Vibrio parahaemolyticus*, an effect which is even more marked in the presence of lactic acid,

suggesting that these may be viable alternatives for extending the preservation time of foods.[26] The same extracts combined were also active and synergic against *Helicobacter pylori*, and it is suggested to manage this bacteria with a diet containing these juices.[27] The results reviewed by above referred literature support to present studies results when compared.

Table 4. MIC and percent growth inhibition of volatile oil, phenolic, non-phenolic fractions of chloroform extract alone and in combination with ciprofloxacin against *C.albicans* by microdilution method.

Concentration (mg/ml)	NP	NP+C	P	P+C	O	O+C	C
0.00015	2	34	8	50	48	66.8	20
0.00030	4	44	16	60	53.2	74.8	32
0.00061	12	56	28	76	68	82.8	44
0.00122	24	58	32	78	75.2	94	52
0.00244	31.2	62.8	36	88	77.2	100	64
0.00488	40	64	42	96	86.8	>100	72
0.00970	52	74	45.2	100	90	>100	84
0.01953	54	82	54.8	>100	100	>100	96
0.03900	61.2	94.8	66	>100	>100	>100	100
0.07800	62	100	72.8	>100	>100	>100	>100
0.15600	70	>100	84	>100	>100	>100	>100
0.31200	72	>100	86	>100	>100	>100	>100
0.62500	80	>100	92	>100	>100	>100	-
1.25000	91.2	>100	100	>100	-	-	-
2.50000	93.2	>100	>100	>100	-	-	-
5.00000	100	>100	>100	>100	-	-	-
10.0000	>100	-	>100	-	-	-	-

NP= non-phenolic fraction, P= phenolic fraction, O= volatile oil, S= standard drug ciprofloxacin, % in= inhibition, - = Dilution was not made in this concentration range for the given sample, MIC= Minimum inhibitory concentration.

Table 5. FIC determination of volatile oil, phenolic, non-phenolic fractions of chloroform extract and standard antibiotic/antifungal drug and FICI determination.

strain	FIC		FIC		FIC		FICI		
	O	S	P	S	NP	S	O	P	NP
P.fluorescens	0.491	0.007	0.062	0.031	0.249	4.000	0.498	0.093	4.249
A.hydrophila	0.500	0.122	0.007	1.000	1.000	512.295	0.622	1.007	513.295
C.albicans	0.124	0.062	0.007	0.248	0.015	2.000	0.186	0.255	2.015

O= volatile oil, P= phenolic fraction, NP= non-phenolic fraction, S= standard (ciprofloxacin for bacterial strain and fluconazole for fungal strain), FIC= Fractional inhibitory concentration, FICI= Fractional inhibitory concentration index.

Conclusion

The experimental data suggests the use of volatile oil and phenolic fraction of the chloroform extract to be used against common fish pathogens *P.fluorescence*, *A.hydrophila* and *C.albicans* in combination with ciprofloxacin and fluconazole respectively to maintain healthy aquaculture and that of the people consuming seafood. The problem of emerging resistance of the micro-organisms against established antibiotics and toxicity can be solved by using these combinations.

Conflict of interest

The authors report no conflicts of interest.

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