Iranian Journal of Microbiology
Volume 7 Number 6 (December 2015) 324-332

ORIGINAL ARTICLE

Nanosilver-marine fungal chitosan as antibiotic synergizers against sepsis fish bacteria

Khouloud Mohamed Barakat[1*], Yousri Mahmoud Gohar[2]

[1]*Microbiology Lababoratoy, Marine Environment Division, National Institute of Oceanography & Fisheries*
[2]*Botany and Microbiology Depart, Faculty of Science, Alexandria University*

Received: June 2015**, Accepted:** October 2015

ABSTRACT

Background and Objectives: Mortality is highly variable within population of cultured fish due to virulent bacteria causing fish septicemia. The use of nano-silver marine fungal chitosan as antibiotic synergisers could be an alternative in the treatment of sepsis fish pathogens.
Materials and Methods: Different bulk chitosan solutions were prepared from the mycelia of four marine fungi (*Aspergillus terreus, Aspergillus flavipes, Tricoderma hamatum* and *Fennellia flavipes*) and used as capping agents for silver nanoparticles. *In vitro*, the antibacterial activity of these preparations was determined against nine fish-sepsis causing bacteria, alone and in combination with nine antibiotics of choice used in aquaculture. Prepared fungal chitosans (Cs_F) were characterized by yield of chistosan obtained, degree of deacetylation and viscosity.
Results and Conclusion: The maximum yield of chitosan (28%) was obtained from *Aspergillus terreus*. *A. terreus* chitosan (Cs_F), silver nanoparticles (AgNPs) and chitosan-silver nanoparticles (Cs_F-AgNPs) showed maximum activity at the minimum inhibitory concentrations average (MIC_{AVG}) 27.2, 18.2 and 7.9 µg/ml, respectively. Combination of Cs_F -AgNPs with amikacin (Ak) and rifampicin (RD) reduced the MIC values by 96 and 94%, respectively, with fractional inhibitory concentration index (FICI) = 0.42 and 0.50 as synergistic effect. It is promising to use Cs_F-AgNPs as enhancing agent in combination with antibiotics for fish sepsis therapy.

Keywords: Marine fungal chitosan, nanosilver, bacterial sepsis, antibiotics, synergy

INTRODUCTION

Outbreaks of bacterial pathologens in dense populations of cultured fish are associated with many stressful conditions (1). Pathogenic bacteria produce similar syndromes represented by hemorrhagic septicemia and ulcerative lesions that are common as disease progresses, with high mortality rates under stress conditions. (2).

Use of the bio-waste chitosans obtained from aquatic organisms has recently been used and commercially available. Preparations of those product in industrial and medical applications depend largely on its physico-chemical characteristics because of difficulties related to seasonal and variable supply of raw materials, as well as difficulties in manufacturing conditions (3). These problems on production of chitosan from crustaseans may be switched by the prepation of chitosan from fungal mycelia (4). The production of chitosan from fungal mycelia is more advantageous than crustacean chitosans concerning its degree of acetylation and viscosity (5). Moreover,

*Corresponding author: Dr. Khouloud Mohamed Barakat, National Institute of Oceanography & Fisheries, Alexandria, Egypt.
Tel: 203 4801553
Fax: 203 4801174.
E-mail: kh2m2@yahoo.com.

distribution and production of the fungal chitosan are more stable than that of crustacean chitosans that ensure providing of maintained stability of final product. Chitosan fungal mycelia which gives medium-low molecular weight are commonly used in many medical-technical applications (6). Reasons make production of new fungal chitosan preparations is highly appreciated. However, the preparation of high pure chitosan from fungal cell wall material has not been accomplished yet (7).

The antimicrobial potential of AgNPs enables their use for medical and veterinary applications because of their good physiochemical properties and low toxicity. The antimicrobial activity of AgNPs against marine bacterial fish pathogens were previously studied (8; 9). Moreover, the extensive use of multi-agents in medical therapy resulted in the emergence of pan-drug resistant bacteria (10). So, attempts have been made to deal with this problem by using more of combined therapy (11). The present study showed the synergistic effects induced by combination of antibiotics of choice with fungal chitosans silver nanoparticles against nine fish sepsis-causing bacteria.

MATERIALS AND METHODS

Fungal cultivation conditions. Four marine fungi *Aspergillus terreus*, *Aspergillus flavipes, Tricoderma hamatum* and *Fennellia flavipes* were isolated from Edko Lake. These strains were identified by Mycological center, Faculty of Science, Assiut University, Egypt; on Chapex medium. One ml spore suspension (10^7 spore ml^{-1}) was prepared from a 7-days-old slants, and inoculated into 100 ml Rose Bengal media in a 250 ml Erlenmeyer flask. The cultures were incubated at 28 ˚C; 120 rpm for 7 days. Fungal dry weights were determined after filtration and drying the biomass pellet at 65 ˚C to achieve constant weight.

Test organisms. Samples were isolated from infected fish by swabbing. Purified colonies on tryptic soya agar (TSA) were subcultured in 5 ml broth medium over-night and identified via biochemical tests (12). Nine bacteria were selected for bioassay: *Aeromonas hydrophila, Edwardsiella tarda, Pasteurella piscicida, Pseudomonas aeruginosa, Streptococcus faecium, Streptococcus iniae, Vibrio ordalli Vibrio anguillarum, Yersinia ruckeri.*

Antibiotics. Six antibiotic groups used in the present study were purchased from local market: Memphis Co. for Pharmaceutical & Chemical Industries Development (CID) Giza, Egypt (Table 1). These antibiotics are enhancing agents in fish therapy protocols (13). Dilutions from the stock antibiotic powders were prepared according to manufacturer's recommendations.

Preparation of fungal chitosan. Fungal chitosan preparing was done as described before in the modified method of Crestini et al. (14). Dry fungal mycelia were ground, suspended with 1 mol^{-1} NaOH solution (1 :30 w/v) and autoclaved at 121 ˚C for 15 min. Alkali-insoluble fractions were collected after centrifugation at 5000 rpm for 15 min, washed with distilled water and recentrifuged to neutrality. The residues were further extracted using 2% acetic acid (1: 40 w/v) at 95 ^{o}C for 8h. The extracted slur-

Table 1: Antibiotics used in the study

Antibiotic group	Antibiotics	Code	Conc. µg/ml
Aminoglycoside	Amikacin	AN	50
	Kanamycin	K	5
Chloramphenicol	Florfenicol	KF	10
Macrolides	Erythromycin	ETH	10
Quinolones	Ciprofloxacin	CIP	5
Rifamycin	Rifampicin	RD	5
Tetracycline	Tetracycline	TE	10
	Oxytetracycline	OT	10
	Minocycline	MC	5

ry was centrifuged at 5000 rpm for 15 min and the insoluble acid discarded. The pH of the supernatant fluids was adjusted to 10 with 2 mol/l NaOH, the solution centrifuged at 5000 rpm for 15 min and the precipitated chitosan was washed with distilled water, 95% ethanol (1 : 20 w/v) and acetone (1 : 20 w/v), respectively and dried at 60 ^{o}C to a constant weight. Physicochemical characterization of obtained fungal chitosan was done. Degree of deacetylation (DDA) was measured and calculated according to an adapted method of Czechowska-Biskup et al. (15), in Central Lab., Faculty of Science, Alexandria University where, viscosity was recorded using Digital Viscometer ST2020R.

Characterization of Cs_F-AgNPs solution was observed through scanning electron microscopy (SEM) (JSM-5300 scanning microscope, JEOL) and used to record chitosan (Cs_F) and Cs_F-AgNPs morphology and size. For this study, the samples were treated first using ultrasonic radiation of the two examined samples, then subsequently gold coating performance before transferring them to the microscope.

Preparation of fungal chitosan-nanosilver. A stable silver nanoparticle was prepared via the wet modified chemical reduction method according to Song et al. (16). Freshly prepared $NaBH_4$ (4×10^{-2} M) solution, as the strong reducing agent, was added to $AgNO_3$ under continuous stirring to reach a constant $AgNO_3/NaBH_4$ molar ratio (1:4). Following this usual preparation method for preparation of Cs_F-AgNPs was carried out using soluble Cs_F as suitable stabilizer and capping agent (100 mL, 0.5 wt %) by solubilization in 1.0 wt% of acetic acid solution (pH ~3.53) under constant stirring. Solution of AgNPs was added to soluble Cs_F under constant stirring for synthesis of the Cs_F-AgNPs solution. Thickened solution of Cs_F-AgNPs obtained were centrifuged and dried at 40 °C under vacuum overnight.

Antimicrobial activities. Antimicrobial activities of fungal chitosan, AgNPs and Cs_F-AgNPs were recorded in the form of minimum inhibitory concentration (MIC) as recommended in the guide of standards of the CLSI (17). MIC was determined, after incubation, by choosing the lowest inhibitory concentration of fungal chitosan or its combinations according to the presence or absence of turbidity. The lower the MICs are, the higher the activity of the tested chitosan or chitosan-nanoparticle is (18).

Fractional inhibitory concentrations index. Antibiotic- Cs_F-AgNPs combinations were applied against the most resistant strains. MICs were determined in three replicates for each combination by broth microdilution according to standards of the CLSI (17). Synergism was defined as a fraction inhibitory concentrations index (FICI) ≤ 0.5, additive effect as an FICI > 0.5 and ≤ 1, indifference effect as an FICI > 1 and ≤ 2 and antagonism effect as an FICI > 4. The FICI for a drug is derived by dividing drug concentration by the control MIC of the drug against the tested organism. FICI for the combinations of two drugs was calculated according to the equation:

$$\text{FIC index} = FIC_{Ab} + FIC_{Cs} = Ab / MIC_{Ab} + Cs / MIC_{Cs}$$

(Ab and Cs refer to antibiotics and chitosans, respectively).

RESULTS

Marine fungal chitosan yield. Four fungal strains were isolated and cultivated using Rose Bengal media. Maximal mycelial dry weight was obtained within 7 days in (g/l) 6.0, 3.5, 5.9 and 9.0. *Aspergillus terreus* gave a maximal yield of chitosan 278 mg g^{-1} dry wt (28% chitosan), while the lowest yield of chitosan was of 12 mg g^{-1} dry wt (1.2% chitosan) for *Aspergillus flavipes* (Fig. 1). Table 2 shows different degree of deacetylation and viscosity of marine fungal strains. The highest percentage of deacetylation and viscosity at 93% and 11.6 centipoises (cP), respectively were

Table 2. Degree of deacetylation and viscosity of different marine fungal Chitosan

Fungal species	Degree of deacetylation (%)	Viscosity (cP)
Aspergillus terreus	93	11.6
Aspergillus flavipes	75	5.1
Fennellia flavipes	62	8.4
Tricoderma hamatum	84	10.3

recorded from the prepared *As. terreus* chitosan.

Chitosan (Cs_F) and Cs_F-AgNPs morphology was characterized through scanning electron microscopy (SEM) showing the reduction of chitosan based silver nanoparticles (Cs_F-nAg) with average sizes 28.4 -39.8 nm (Fig. 2).

Bioactivity of chitosans, nanosilver and chitosan-nanosilver. Using *Aspergillus terreus* chitosan (CS_F) revealed its strong antibacterial activity against resistant strains. One percent chitosan solution could inhibit sepsis bacteria, showing maximum average of MIC_{AVG} 27.2 µg/ml. Lower MIC_{AVG} were recorded with *Fennela flavepis* chitosan 292.0 µg/ml (Fig. 3). No activity was obtained using silver-nanoparticle (Fig. 4). The capping agent silver nanoparticles showed more challenged activity using fungal chitosans. *Aspergilus terreus* - CS_F-AgNPs showed antibacterial activity with maximum average of 7.9 µg/ml, while, lower MIC_{AVG} were recorded with *Fennela flavepis* chitosan at 195.9 µg/ml (Fig. 5).

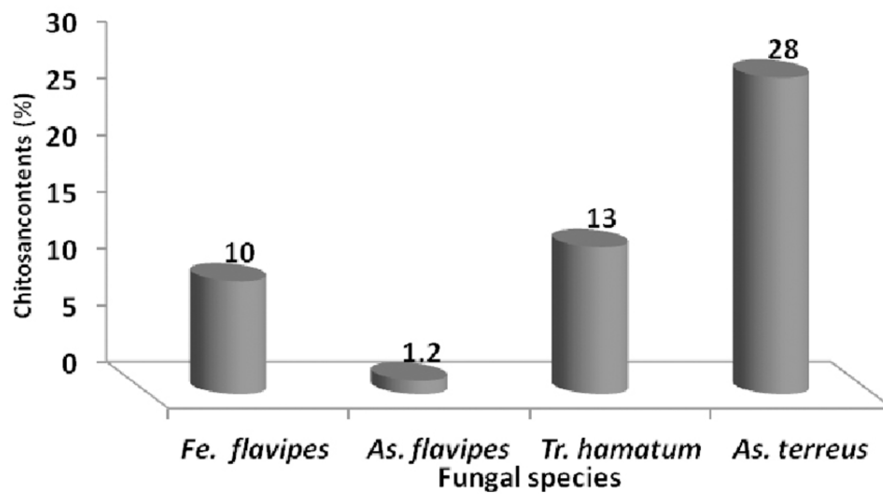

Fig. 1. Chitosan yield produced from four different fungal species.

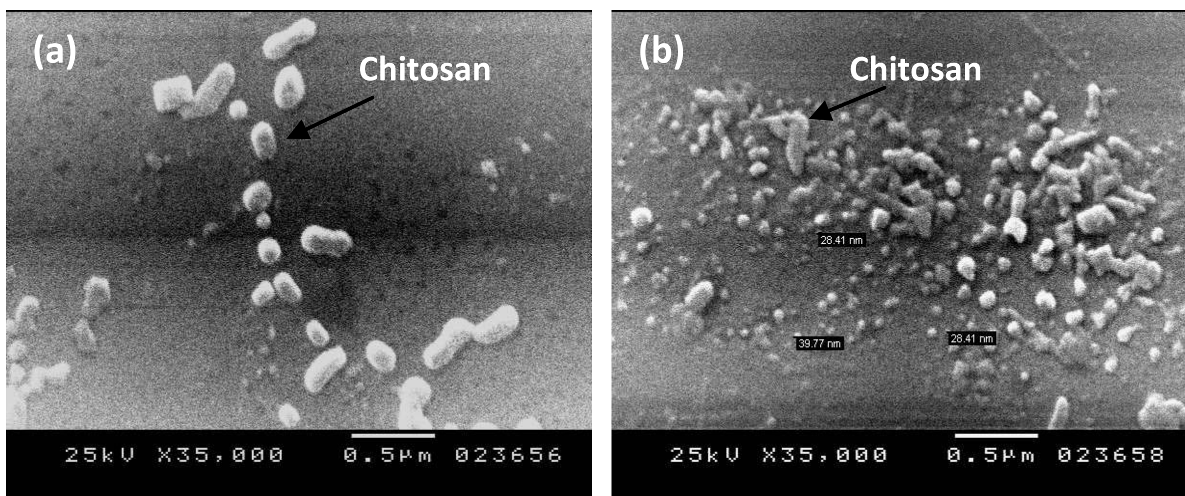

Fig. 2. Scan electron microscope (SEM) showing fungal chitosan particles (Cs_F) (a) and chitosan based silver nanoparticles (Cs_F-nAg) (b).

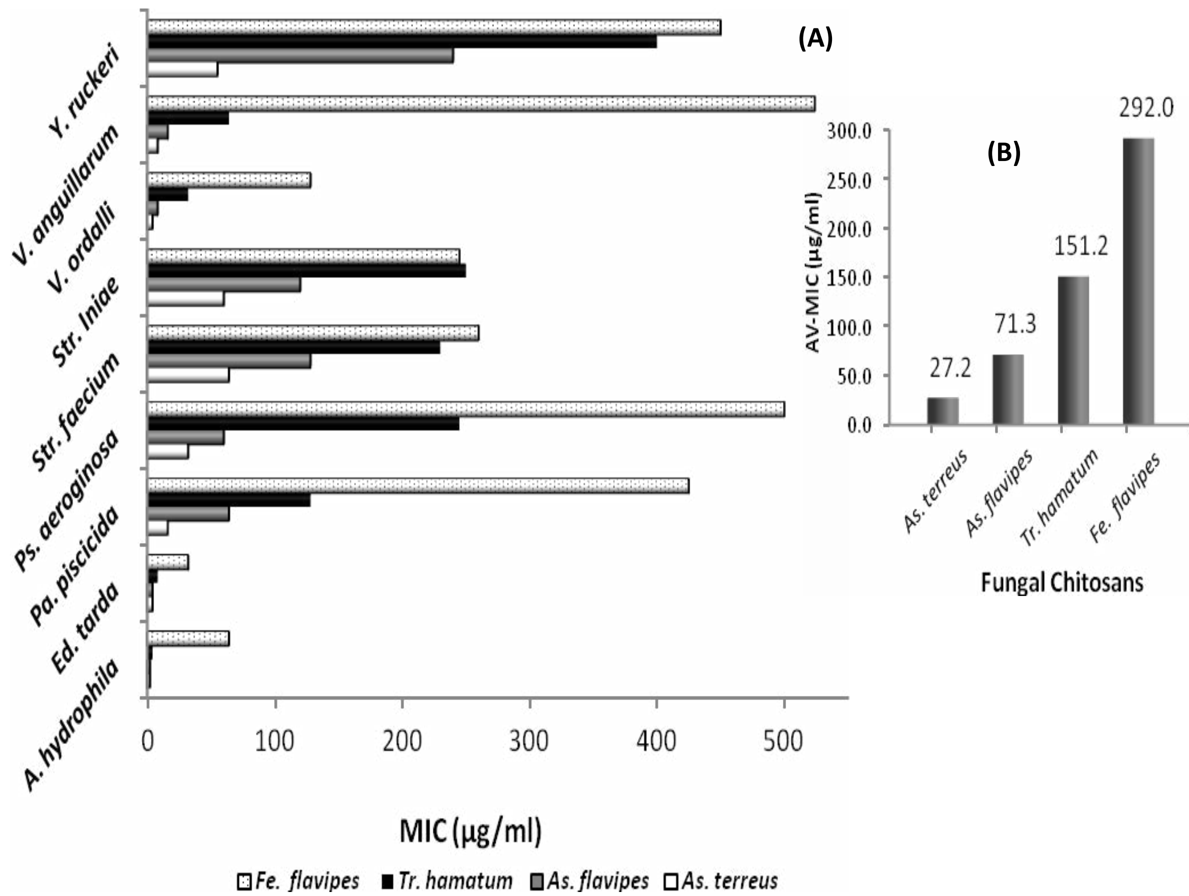

Fig. 3. Minimum inhibitory concentration of different fungal chitosans against sepsis-causing bacteria (A) and MIC_{AVG} of different fungal chitosans (B).

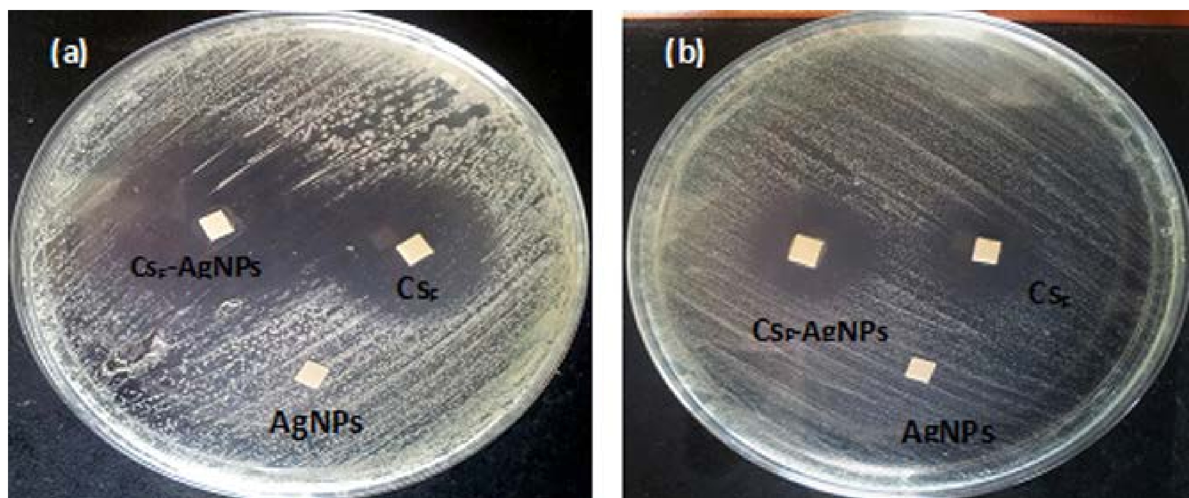

Fig. 4. Antibacterial activity of *A. terreus* chitosan based silver nanoparticles (Cs_F-AgNPs), *A. terreus* chitosan (Cs_F) and silver nanoparticles AgNPs against *A. hydrophilla* (most sensitive strain) (a) and *Ps. aeruginosa* (most resistant strain) (b)

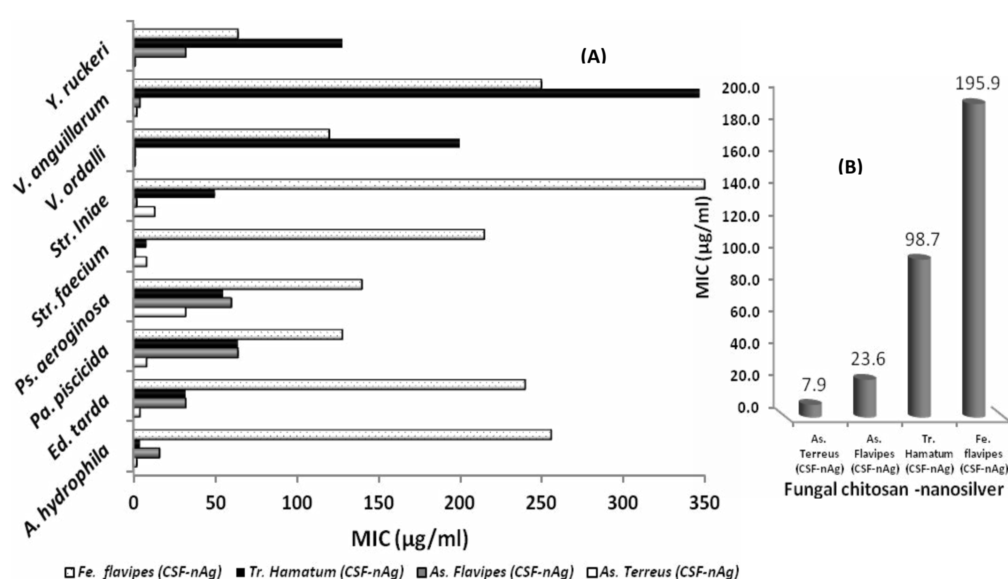

Fig. 5. Minimum inhibitory concentration of different fungal chitosans nano-silver against sepsis bacteria (A) and MIC_{AVG} of different fungal chitosans nano-silver (B).

Combinations and FICI. Using microdilution assay, sepsis bacteria treated with antibiotics combined with fungal chitosan silver nano-particle showed synergistic action with amikacin & rifampicin at maximum MIC 11.4±0.6 &16.4±0.8 µg/ml (Table 3) and the FICI were 0.42 and 0.50 (Fig. 6), respectively. The additive effects were recorded using kanamycin, florfenicol, erythromycin, ciprofloxacin tetracycline and minocycline -CS_F-AgNPs with FICI range from 0.63 – 0.86. The indifference effect was recorded at 1.04 for oxytetracycline-CS_F-AgNPs.

From the synergistic point, combination of AgNPs-CS_{Sq} with amikacin (Ak) and rifampicin (RD) reduced the MIC value from 256 µg/ml to a much lower values 11.4 and 16.4 µg/ml, respectively, against sepsis bacteria. Finally, the best formulae obtained from these previous results were:

Amikacin – CS_F-AgNPs & Rifampicin– CS_F-AgNPs

Table 3. Minimum inhibitory concentration of antibiotics - fungal chitosan – AgNPs combinations against sepsis bacteria

Antibiotic	Combination	MIC (µg/ml)*									
		A. hydrophila	*Ed. tarda*	*Pas. piscicida*	*Ps. Aeruginosa*	*Str. Faecium*	*Str. iniae*	*V. ordalli*	*V. anguillarum*	*Y. ruckeri*	AV-AB -CS_F-AgNPs
***As. terreus* chitosans AgNPs**		2±0.1	4±0.2	8±0.4	32±1.6	8±0.4	13±0.7	1±0.1	2±0.1	1±0.1	7.9±0.4
Amikacin	**AN-CSF-AgNPs**	9±0.5	8±0.4	8±0.4	16±0.8	22±1.1	0	16±0.8	8±0.4	16±0.8	11.4±0.6
Kanamycin	**K-CSF-AgNPs**	9±0.5	32±1.6	12±0.6	16±0.8	48±2.4	18±0.9	48±2.4	16±0.8	24±1.2	24.8±1.2
Florfenicol	**KF-CSF-AgNPs**	9±0.5	16±0.8	32±1.6	16±0.8	32±1.6	8±0.4	24±1.2	34±1.7	32±1.6	22.6±1.1
Erythromycin	**ETH-CSF-AgNPs**	10±0.5	32±1.6	34±1.7	22±1.1	34±1.7	12±0.6	48±2.4	24±1.2	34±1.7	27.8±1.4
Ciprofloxacin	**CIP-CSF-AgNPs**	7±0.4	12±0.6	24±1.2	12±0.6	22±1.1	11±0.5	64±3.2	32±1.6	64±3.2	27.6±1.4
Rifampicin	**RD-CSF-AgNPs**	9±0.5	16±0.8	24±1.2	8±0.4	16±0.8	9±0.5	22±1.1	12±0.6	32±1.6	16.4±0.8
Tetracyclin	**TE-CSF-AgNPs**	11±0.6	32±1.6	6±0.3	16±0.8	22±1.1	7±0.4	64±3.2	48±2.4	48±2.4	28.2±1.4
Oxytetracyclin	**OT-CSF-AgNPs**	11±0.6	64±3.2	16±0.8	24±1.2	48±2.4	12±0.6	34±1.7	32±1.6	32±1.6	30.3±1.5
Minocyclin	**MC-CSF-AgNPs**	11±0.6	64±3.2	16±0.8	32±1.6	32±1.6	11±0.5	22±1.1	12±0.6	16±0.8	24.0±1.2

* Standard Error of Data at 5%

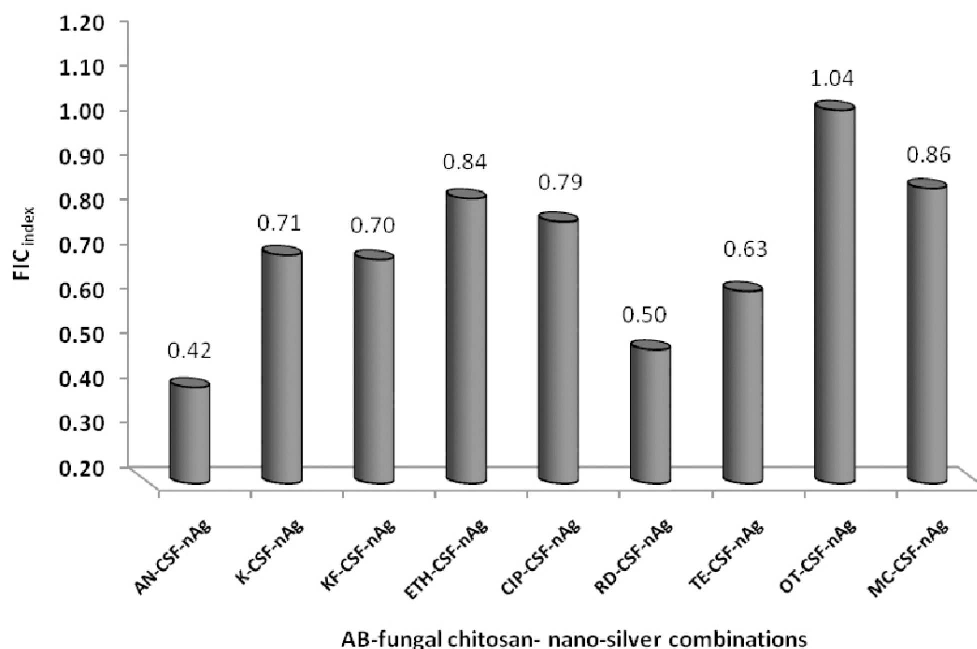

Fig. 6. Average of fractional inhibitory concentration index (FICI) of antibiotics–fungal chitosan–AgNPs combination against sepsis fish isolates

DISCUSSION

Chitosans are polymers combining a group of physicochemical and biological characteristics, which allow wide scope of applications (19). Previous studies were concentrated on the biological activity of chitosan from exoskeletons of fungi with spectrum of activity against pathogens and lower toxicity toward animal cells (20; 21; 22).

Four fungal chitosans capped agents' for silver nanoparticles were prepared and used for antibacterial activity against some sepsis fish pathogens. These preparations were carried out separately and in combination with antibiotics. *A. terreus* appeared to have a greater potential to be used for preparation of chitinous material due to a more abundant biomass production. Maximum yield for *A. terreus* (9.0 g/L) was obtained on the 6th day after inoculation and shown to give a maximal yield of chitosan 28%. Fungal chitosan accumulation was found at a different stage of cell growth but the late exponential phase produced the most (2). *A. terreus* chitosan yield were higher than that previously obtained by different *A. niger* strains ranged from 17.2- 26.1% (23). Barely similar to our results, the highest yield of chitosan from the dry mycelia of *Penicillium waksmanii* was 29.7% (24).

Our results revealed that the prepared *A. terreus* chitosan (CS_F) could inhibit sepsis fish pathogens completely with maximum average of MIC 28.7 µg/ml. Logesh et al. (22), showed maximum activity of marine fungal chitosan at range of 32 - > 256 µg/ml against *V. cholera, E. coli* and *S. aureus.* Fungal chitosan was not a macromolecule polymer, it seems to be able to pass the outer membrane of bacteria, since this membrane functions as an efficient outer permeability barrier against other chitosan macromolecules (25).

Fungal chitosan capped with silver nanoparticle CS_F-AgNPs showed antimicrobial action at 7.9 µg/ml. The effect of the synthesized silver/chitosan nanocomposites showed high antibacterial activities against Gram-positive bacteria (26). Combinations of different antibiotics as antimicrobials in fish therapy are previously reported by Inglis et al. (27), showed no effect of trimethoprim and quinolones against Gram-negative fish pathogens in preventing emergence of resistance. However, combination of natural biowastes with non effective drugs may produce synergistic action. Our results revealed synergy of Cs_F-AgNPs in combination with amkacin and rifampacin against sepsis fish pathogens by 11.4 and 16.4 µg/ml. Compared to our results, the antimicrobial

synergic effect of chitosan with antibiotics against the bacterial flora of fish showed MICs ranged from 27 to 125 µg/ml (28). Also, the synergic effect of chitosan with sulfamethoxazole against *P. aeruginosa* reduced MIC from 64 to 8 µg/ml (29). Decker et al. (30) proposed a synergistic chlorhexidine/chitosan combination yielded a strong CFU reduction much stronger than chlorhexidine alone. Therefore, our results reveal the importance of antibiotics-fungal chitosan - silver nanoparticles combinations as they have strong antibacterial activity against resistant sepsis fish pathogen.

Pathogens treated with antibiotics combined with Cs_F-AgNPs showed synergistic action at maximum FIC_{index} 0.42 and 0.50. Combination studies of leaf extract fractions along with ciprofloxacin and fluconazole showed activities against common fish pathogens where maximum FIC_{index} ranged from 0.007 – 0.50 (31). Many authors studied different synergic FIC_{index} effect against fish pathogens (FIC less-than or equal to 0.5) (32, 33). FIC_{index} of chitosan oligosaccharide proves to be better combination for sulfamethoxazole against *P. aeruginosa* at FIC_{index} 0.375 (29). While, the combination of three chitosans and bacteriocin (divergicin M35) appeared to have an additive effect against *Listeria monocytogenes,* as determined by $FIC_{index} = 1$ (34).

Thus we concluded that fungal chitosan silver nanoparticles combined with amikacin and rifampacin, *in vitro*, could be a promising alternative against sepsis fish pathogens. The final synergistic formula against these isolates has a positive role in preventing the resistance mechanism by these virulent bacteria. Also, these data encourage further studies with fungal chitosan and other antimicrobial classes *in vivo* using animal experiments to validate these interesting findings.

REFERENCES

1. Gomez DK, Sato J, Mushiake K, Isshiki T, Okinaka Y. Naka T. CR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *J Fish Dis* 2004; 27: 603-608.
2. Gomez DK, Lim DJ, Baeck GW, Youn HJ, Shin NS, Youn HY, et al. Detection of betanodaviruses in apparently healthy aquarium fishes and invertebrates. *J Vet Sci* 2006; 7: 369–374.
3. Nwe N, Furuike T, Tamura H. The mechanical and biological properties of chitosan scaffolds for tissue regeneration templates are significantly enhanced by chitosan from *Gongronella butleri. Materials* 2009; 2: 374-398.
4. Nwe N, Furuike T, Tamura H. Selection of biopolymer base on attachment, morphology and viability of fibroblast NIH/3T3 cells for the development of a biodegradable tissue regeneration template: alginate, bacterial cellulose and gelatin. *Process Biochemistry* 2010; 45: 457-466.
5. Jang M-K, Kong B-G, Jeong Y-I, Lee CH, Nah J-W. Physicochemical characterization of a-chitin, b-chitin and g-chitin separated from natural resources. *J Pol Sc* 2004; Part A: Polymer Chemistry 42: 3423–3432.
6. Azuma K, Ifuku S, Osaki T, Okamoto Y, Minami S. Preparation and biomedical applications of chitin and chitosan nanofibers. *J Biomed Nanotechnol* 2014; 10(10): 2891-2920.
7. Nwe N, Furuike T, Tamura H. Production, Properties and Applicationsof Fungal Cell Wall Polysaccharides: Chitosan and Glucan. *Adv Polym Sci* 2011; 244: 187–208.
8. Krishnaraj C, Jagan EG, Rajasekar S, Selvakumar P, Kalaichelvan PT, Mohan N. Synthesis of silver nanoparticles using *Acalypha indica* leaf extracts and its antibacterial activity against water borne pathogens was studies. *Colloids Surf B Biointerfaces* 2010; 76: 50-56.
9. Velmurugan P, Iydroose M, Lee SM, Cho M, Park JH, Balachandar V, et al. Synthesis of silver and gold nanoparticles using cashew nut shell liquid and its antibacterial activity against fish pathogens. *Indian J Microbiol* 2014; 54:196-202.
10. Falagas ME, Bliziotis IA, Kasiakou SK, Samonis G, Athanassopoulou P, Michalopoulos A. Outcome of infections due to pandrug-resistant (PDR) Gram-negative bacteria. *BMC Infect Dis* 2005; 5: 24-30.
11. Ling TKW, Xiong J, Yu Y, Lee CC, Ye H, Hawkey PM. Multicenter antimicrobial susceptibility survey of Gram-negative bacteria isolated from patients with community-acquired infections in the People's Republic of China. *Antimicrob Agents Chem* 2006; 50: 374-378.
12. Steel C (1993). Manual for identification of medical bacteria, 3rd edition. Cambridge University Press, U.S.A.
13. EAEMP (1997). The European Agency for Evaluation of Medical Products, veterinary medicine evaluation unit. Committee for veterinary medicine products, summary report 2 (Florfenicol, extension to fish). West circus, canary wharf, London, E 14 4HB, UK.
14. Crestini C, Kovac B, Giovannozzi-Sermanni G. Production and isolation of chitosan by submerged and solid state fermentation from *Lentinus edodes. Biotechnol Bioeng* 1996; 50: 207–10.

15. Czechowska-Biskup R, Jarosińska D, Rokita B, Ulański P, Rosiak JM. Determination of degree of deacetylation of chitosan - comparision of methods. *Progress Chem Appl Chitin its Derivatives* 2012; 17: 5-20.
16. Song KC, Lee MS, Park TS. Preparation of colloidal silver nanoparticles by chemical reduction method. *Korean J Chem Eng* 2009; 26:153–155.
17. Richard S, Lynn S-M, Avery CG (2007). Antimicrobial susceptibility testing protocols. New York: CRC Press.
18. Andrews JM BSAC standardized disc susceptibility testing method. *J Antimicrob Chemoth* 2001; 48(S1): 43-57.
19. Takemono K, Sunamoto J, Askasi M (1989). Polymers and medical care. Mita. Tokyo, chapter IV.
20. Wu T, Zivanovic S, Draughon FA, Conway WS, Sams CE. Physicochemical properties and bioactivity of fungal chitin and chitosan. *Food Chem* 2005; 53: 3888-3894.
21. Eweis M, Elkholy SS, Elsabee MZ (2006) Antifungal efficacy of chitosan and its thiourea derivatives upon the growth of some sugar beet pathogens. *Int J Biol Macromol* 38: 1-8.
22. Logesh AR, Thillaimaharani KA, Sharmila K, Kalaiselvam M, Raffi SM. Production of chitosan from endolichenic fungi isolated from mangrove environment and its antagonistic activity. *Asian Pacific J Trop Biomed* 2012; 2: 140-143.
23. Kumaresapillai N, Bashab RA, Sathisha R. Production and evaluation of chitosan from *Aspergillus niger* MTCC Strains. *Iran J Pharm Res* 2011; 10: 553-558.
24. Ebrahimzadeh MA, Chabra A, Gharaei-Fathabad E, Pourmorad F. Preparation of chitosan from *Penicillium* spp. and determination of their degree of deacetylation. Ind. *J Biotechnol* 2013; 12: 231-235.
25. Mohy- Eldin MS, Soliman EA, Hashem AI, Tamer TM. Antibacterial activity of chitosan chemically modified with new technique. *Trends Biomater Artif Organs* 2008; 22: 125-137.
26. Shameli K, Ahmad MB, Zargar M, Yunus WM, Ibrahim NA, Shabanzadeh P, Moghaddam MG. Synthesis and characterization of silver/ montmorillonite/chitosan bionanocomposites by chemical reduction method and their antibacterial activity. *Int J Nanomedicine* 2011; 6: 271–284.
27. Inglis V, Cafini M, Yoshida T. The interaction of trimethoprim and quinolones against gram-negative fish pathogens. *J Appl Bacteriol* 1995; 79:135-140.
28. Schelegueda LI, Gliemmo MF, Campos CA. Antimicrobial synergic effect of chitosan with sodium lactate, nisin or potassium sorbate against the bacterial flora of fish. *J Food Res* 2012; 1: 272- 281.
29. Tin S, Sakharkar KR, Lim CS, Sakharkar MK. Activity of chitosans in combination with antibiotics in *Pseudomonas aeruginosa*. *Int J Biol Sci* 2009; 5: 153–160.
30. Decker E-M, von Ohle C, Weiger R, Wiech I, Brecx MA. Synergistic chlorhexidine/chitosan combination for improved antiplaque strategies. *J Period Res* 2005; 40: 373-377.
31. Bharti V, Vasudeva N, Dhuhan JS. Combination studies of *Oreganum Vulgare* extract fractions and volatile oil along with ciprofloxacin and fluconazole against common fish pathogens. *Adv Pharm Bull 2*013; 3: 239–246.
32. Lee E-M, Choi M-J, Lee S-J, Park S-C. Pharmacodynamics of florfenicol alone and in combination with amoxicillin or cefuroxime against pathogenic bacteria of fish origin. *Korean J Vet Res* 2010; 50 : 279-284.
33. Prasad VGNV, Swamy PL, Rao TS, Rao GS Antibacterial synergy between oxytetracycline and selected polyphenols against bacterial fish pathogens. *Inter J Vet Sci* 2013; 2: 71-74.
34. Benabbou R, Zihler A, Desbiens M, Kheadr E, Subirade M, Fliss I. Inhibition of *Listeria monocytogenes* by a combination of chitosan and divergicin M35. *Can J Microbiol* 2009; 55:347-355.