한국환경농학회지 제28권 제1호 (2009)
*Korean Journal of Environmental Agriculture* Vol. 28, No. 1, pp. 75-81

연구보문

# *In Vitro* Antilisterial Potential of a Marine Isolate of *Aspergillus* sp. Collected from the South Coast of Korea

Vivek K. Bajpai, Sun Chul Kang*

Department of Biotechnology, Daegu University, Kyoungsan, Kyoungbook 712-714, Republic of Korea

(2009년 3월 10일 접수, 2009년 3월 24일 수리)

**ABSTRACT: This study was carried out to assess the antilisterial potential of ethyl acetate (EtOAc) extract of a marine isolate of *Aspergillus* sp. The *in vitro* antilisterial efficacy of ethyl acetate extract was examined using disc diffusion, minimum inhibitory concentration (MIC) determination, cell viable count and scanning electron microscopic (SEM) methods against the employed strains of *Listeria monocytogenus*. The ethyl acetate extract (300 μg disc$^{-1}$) exhibited a promising antilisterial effect as diameters of inhibition zones against *L. monocytogenes* ATCC 19111, 19116, 19118, 19166 and 15313, which were found in the range of 11-17 mm along with their MIC values ranging from 125 to 1000 μg ml$^{-1}$, respectively. Also the EtOAc extract had strong detrimental effect on the viable count of the tested *L. monocytogens* ATCC 19166. Furthermore, scanning electron microscopic (SEM) study demonstrated potential detrimental effect of ethyl acetate extract on the morphology of *L. monocytogenes* ATCC 19116 at the used MIC concentration. These findings strongly support the role of ethyl acetate extract of a marine isolate of *Aspergillus* sp. as an antiliterial potential.**

**Key Words:** *Aspergillus* sp., marine fungus, antilisterial potential, ethyl acetate extract, *Listeria monocytogenes*

## INTRODUCTION

*Listeria monocytogenes* is an important food-borne pathogen due to the severity of infection with a high mortality rate. There are intense researches on natural antimicrobial derivatives from animals, plants and microorganisms. These substances possess high potential to reduce pathogenic bacteria[1]. The gram-positive facultative intracellular food-borne pathogen *Listeria monocytogenes* is potentially pathogenic and associated with serious localized and invasive infections in humans and variety of other vertebrates, including domesticated and wild birds and mammals[2]. *Listeria* organisms have been isolated from a variety of sources such as humans, animals and foods including dairy and meat products and found to have numerous major outbreaks worldwide[3].

*연락저자:
Tel: +82-53-850-6553 Fax: +82-53-850-6559
E-mail: sckang@daegu.ac.kr

Investigations of food-borne outbreaks have provided compelling evidences that a febrile gastroenteritis syndrome may indeed be the main clinical manifestation of *L. monocytogenes* infection[4]. Similarly, episodes of gastroenteritis in humans occur in the form of outbreaks with very short incubation periods and high attack rates among immunocompetent adults[5], consistent with the ingestion of very high dose of bacteria, as estimated for one of these outbreaks, caused by the consumption of heavily contaminated chocolate milk[4].

*L. monocytogenes* is capable of survival and growth under widely varying environmental conditions and consequently, contaminate the raw materials used in the preparation of industrially processed foods and the production plants as well[6]. Further, this organism is able to invade and multiply in a wide range of professional and non-professional phagocytic mammalian cells[7]. As contaminated food is the major source of infection in both epidemic and sporadic cases[2], the

gastrointestinal tract is thought to be the primary site of entry of pathogenic *Listeria* organisms into the host[4]. In few decades, *L. monocytogenes* has been established as an important food-borne pathogen due to a series of epidemic outbreaks by listeriosis[8]. Thus, nowadays it is recognized as an important human pathogen, infecting specially immunodepressed patient, elderly, pregnant and newborns[9]. Besides, researchers in immunology were interested in *L. monocytogenes* long before its importance as a risk to public health and food safety was recognized. Also, the experimental model of *Listeria* infection in the mouse has made a significant contribution to understand the cellular immune response in humans[10].

Furthermore, a variety of different chemicals and synthetic compounds have been used as antimicrobial agents to inhibit *L. monocytogenes* growth in food and human systems. Some synthetic antimicrobials can also cause environmental pollution owing to their slow biodegradation in the environment. Resistance to traditional antimicrobials, the limitation of the number of bactericides, and increasing public concern regarding severe illness of listeriosis have also increased the need for the development of new safe and biodegradable alternatives (i.e. the so-called "natural" antilisterial agents). Therefore, it is indeed required the use of naturally occurring antimicrobial agents to inhibit *Listeria* organisms that cause severe infections in foods and human beings. Owing to the source limitation of terrestrial organisms like plants and microbes, most of which have been well investigated chemically and biologically, scientists have begun to extend their attention to those living in oceans since the early 1960s. Antimicrobial substances from natural sources like microorganisms have been investigated to achieve higher levels of human safety standards[11]. Moreover, the whole population of marine microorganisms is being thought to possess a strikingly bigger biodiversity than those of animal and plant kingdoms. This is why marine microbes are currently receiving much more attention highlighting that some marine microbial products have become potential especially for their different bioactivities[12].

Previously we reported the antidermatophytic potential of the ethyl acetate extract of *Aspergillus* sp.[13]. In the present investigation, we assessed the antilisterial efficacy of ethyl acetate extract of a marine isolates of *Aspergillus* sp. against a panel of *Listeria monocytogenes* as an antilisterial potential.

## MATERIALS AND METHODS

### Microorganisms

The bacterial strains used in this study were *Listeria monocytogenes* ATCC 19111, 19116, 19118, 19166 and 15313, which were obtained from Korean Agricultural Culture Collection (KACC), Suwon, Republic of Korea. All the strains were maintained on BHI agar (brain heart infusion, Difco) at 4℃ and grown in BHI broth at 37℃ for 24 h.

### Preparation of ethyl acetate extract

*Aspergillus* species are common and widespread in nature. The native habitat of *Aspergillus* species are in soil, decaying wood, hay, and grains undergoing microbiological deterioration and invade all types of organic substrates whenever the conditions are favorable for their growth. *Aspergillus* species are ubiquitous molds that cause allergies (brewer's lung) in otherwise healthy people and serious sinusitis, pneumonia, and fungemia in immunocompromised individuals. The major factors that predispose to *Aspergillus* infection are neutropenia and corticosteroids. These saprophytic fungi sporulate and produce abundant conidia (asexual spores) that are readily aerosolized. *Aspergillus* species are transmitted by airborne conidia, and the lung is the major portal of entry. *Aspergillus* species produce several virulence factors, including adhesins, antioxidants, enzymes, and toxins.

The fresh mycelium plugs of *Aspergillus* sp. grown on PDA medium at 24±1℃ for 5-6 days were inoculated separately into 3 liter Erlenmeyer flasks containing (1.5 L×10) potato dextrose broth (PDB) medium. After 12 days of incubation at 24±1℃, 12 liter of culture liquid was extracted with EtOAc followed by incubation for 2-3 days at 24±1℃ and 150 rev/min on a rotary shaker. Evaporation of the solvent from the extract *in vacuo* gave a residue, which was subjected to the antilisterial potential.

### Disc diffusion assay

The agar diffusion method was used for antilisterial activity assay[14]. Petri plates were prepared by pouring 20 ml of BHI medium and allowed to solidify. Plates

were dried and 0.1 ml of standardized inoculum suspension containing $10^7$ CFU/ml was poured and uniformly spread. The inoculum was allowed to dry for 5 min. Whatman No. 1 sterile filter paper discs (6 mm diameter) were impregnated with 300 μg $disc^{-1}$ EtOAc extract of *Aspergillus* sp. Negative controls were prepared using the same solvent employed to dissolve the extract. The discs were allowed to dry at room temperature. The plates were incubated at 37℃ for 24 h. Antilisterial efficacy of EtOAc extract of *Aspergillus* sp. was evaluated by measuring the diameter of the zones of inhibition against the tested bacteria. Each assay in this experiment was replicated three times.

### Determination of minimum inhibitory concentration

Minimum inhibition concentrations (MICs) of EtOAc extract of *Aspergillus* sp. were determined by two-fold serial dilution method[15]. The tests of EtOAc extract were incorporated into BHI broth medium to get a concentration of 1000 μg $ml^{-1}$ and serially diluted to achieve the concentrations of 500, 250, 125 and 62.5 μg $m^{-1}$, respectively. 10 μl of standardized suspension of tested organism was transferred to each tube. The control tubes contained only bacterial suspension, were incubated at 37℃ for 24 h. The lowest concentrations of the test samples, which did not show any growth of tested organism after macroscopic evaluation, were determined as MICs, which were expressed in μg $ml^{-1}$.

### Effect of EtOAc extract on viable counts

Active culture for viable count assay was prepared in BHI broth medium[16]. One ml of active stock solution was transferred to 4 ml of BHI broth. Treated culture was kept under microaerobic conditions at 37°C for 2 h. The culture was then centrifuged at 10,000 rpm for 10 min. The pellet was retained and resuspended with 1 ml of phosphate-buffered saline. For viable counts, each of the tubes containing resuspended bacterial suspension (approximately $10^7$ CFU/ml) of *L. monocytogenes* ATCC 19166 was inoculated with the minimum inhibitory concentration of the ethyl acetate extract of *Aspergillus* sp. in 10 ml BHI broth, and kept at 37°C. Samples for viable cell counts were taken out at 0, 20, 40, 60, 80, 100 and 140 min time intervals. The viable plate counts were monitored as followed: After incubation, 1 ml of the resuspended culture was diluted into 9 ml buffer peptone water, there by diluting it 10-fold. 0.1 ml sample of each treatment was diluted and spread on the surface of BHI agar. The colonies were counted after 24 h of incubation at 37°C. The controls were inoculated without extract for *L. monocytogenes* ATCC 19166 with same experimental condition as mentioned above.

### Scanning electron microscopic (SEM) analysis

To determine the efficacy of ethyl acetate extract of *Aspergillus* sp. on the morphology of *L. monocytogenes* 19166, SEM study was performed using minimum inhibitory concentration of ethyl acetate extract. Controls were prepared without extract. Further, to observe the morphological changes, the method of SEM was modified from Kockro method[17]. The bacterial sample was washed gently with 50 mM $l^{-1}$ phosphate buffer solution (pH 7.2), fixed with 2.5 g 100 $ml^{-1}$ glutaraldehyde and 1 g 100 $ml^{-1}$ osmic acid solution. The specimen was dehydrated using sequential exposure per ethanol concentrations ranging from 30-100%. The ethanol was replaced by tertiary butyl alcohol. After dehydration, the specimen was dried with $CO_2$. Finally, the specimen was sputter-coated with gold in an ion coater for 2 min, followed by microscopic examinations (S-4300; Hitachi).

### Statistical analysis

The data obtained for antilisterial activity of ethyl acetate extract of *Aspergillus* sp. were statistically analyzed and mean values were calculated. A Student's *t*-test was computed for the statistical significance ($p>0.05$) of the results.

## RESULTS AND DISCUSSION

The *in vitro* antilisterial efficacy of EtOAc extract of *Aspergillus* sp. against the employed bacteria was qualitatively assessed by the presence or absence of inhibition zones and zone diameters. According to the results given in Table 1, a total of five listerial strains were tested. The EtOAc extract exhibited promising antilisterial effect against all the listerial strains at 300 μg $disc^{-1}$. In particular the EtOAc extract exhibited a potent antilisterial effect as the

values of diameter inhibition zones of 15, 17, 15, 11 and 14 mm against *Listeria monocytogenes* ATCC 19111, 19166, 19116, 15313 and 19118, respectively. Strains ATCC 19111, 19166, 19116 and 19118 were found highly susceptible to the EtOAc extract. However, *L. monocytogenes* ATCC 15313 was inhibited moderately by the EtOAc extract. The blind control did not inhibit the growth of any of the strains tested.

As shown in Table 2, the MIC values for the EtOAc extract against the tested strains of *L. monocytogenes* were found in the range of 125 to 1000 μg $ml^{-1}$. The listerial strains ATCC 19111, 19166 and 19116 were found highly susceptible to EtOAc extract. Ethyl acetate extract exhibited the lowest MIC value (125 μg $ml^{-1}$) against *L. monocytogenes* ATCC 19166. Also, a potent antilisterial effect of EtOAc extract was observed against the strains ATCC 19111, 19116 and 19118 with their respective MIC values of 250, 250 and 500 μg $ml^{-1}$. Only one of the strains *L. monocytogenes* ATCC 15315 displayed less sensitivity to the ethyl acetate extract of *Aspergillus* sp. with MIC value of 1000 μg $ml^{-1}$.

Based on the susceptibility, further study was carried out to evaluate the effect of ethyl acetate extract of *Aspergillus* sp. on the viable count of *L. monocytogenes* ATCC 19166. The ethyl acetate extract demonstrated the reduced viability on the growth of *L. monocytogenes* ATCC 19166 at MIC concentration. At 40 min exposure, 80-90% inhibition of *L. monocytogenes* ATCC 19166 was observed. Also the steep decline in CFU numbers was observed at 20 min exposure against *L. monocytogenes* ATCC 19166. Exposure of 60 min of the ethyl acetate extract of *Aspergillus* sp. revealed complete inhibition of CFU numbers against *L. monocytogenes* ATCC 19166 at MIC concentration (Table 3).

Furthermore, elaborative study of SEM was also carried out to visualize the effects of ethyl acetate extract on the morphology of *L. monoctytogenes* 19166 and demonstrated altered cell morphology of *L. monoctytogenes* 19166 as compared to control group. (Fig. 1). Control cells in the absence of the extract showed a regular, smooth surface (Fig. 1a). In contrast, cells inoculated with ethyl acetate extract at MIC concentration (125 μg $ml^{-1}$) revealed severe detrimental effect on the morphology of cell membrane,

**Table 1. Antilisterial activity of ethyl acetate (EtOAc) extract of a marine isolate of *Aspergillus* sp.**

| Microorganism | Diameter of zones of inhibition (mm)[a] | |
|---|---|---|
| | EtOAc extract[b] | SM (standard)[c] |
| *Listeria monocytogenes* ATCC 19111 | 15±1.1 | nd[d] |
| *Listeria monocytogenes* ATCC 19166 | 17±1.3 | 14.0±0.7 |
| *Listeria monocytogenes* ATCC 19116 | 15±1.2 | 15.3±0.5 |
| *Listeria monocytogenes* ATCC 19313 | 11±1.7 | nd |
| *Listeria monocytogenes* ATCC 19118 | 14±1.1 | 15.2±0.5 |

[a] Diameter of inhibition zones of ethyl acetate extract including diameter of disc 6 mm (tested concentrations: 300 μg $disc^{-1}$); [b] Ethyl acetate extract of *Aspergillus* sp.; [c] Streptomycin; [d] Antilisterial activity not detected.
* Values are given as mean ± Standard Deviation (S.D.) of triplicate experiment.
* EtOAc as negative control had no antilisterial effect.

**Table 2. Minimum inhibitory concentration of ethyl acetate (EtOAc) extract of a marine isolate of *Aspergillus* sp.**

| Microorganism | MIC[a] |
|---|---|
| | EtOAc extract[b] |
| *Listeria monocytogenes* ATCC 19111 | 250 |
| *Listeria monocytogenes* ATCC 19166 | 125 |
| *Listeria monocytogenes* ATCC 19116 | 250 |
| *Listeria monocytogenes* ATCC 19313 | 1000 |
| *Listeria monocytogenes* ATCC 19118 | 500 |

[a] Mminimum inhibitory concentration (values in μg $ml^{-1}$); [b] Ethyl acetate extract of *Aspergillus* sp.

showing disruption and lysis of the membrane integrity (Fig. 1b and 1c). Infact, initial exposure of ethyl acetate extract to *L. monoctytogenes* 19166 revealed large surface collapse and wrinkled abnormalities on the morphology of the cells along with some small clefts (Fig. 1d). Such morphological features in bacterial cells might be due to the lysis of outer membrane and the transformation by weak peptidoglycan followed by the loss of cellular electron dense material on surface of the treated cells, resulting in the release of inner cell materials as evident by our previous findings and the literature reported by others[16,18].

In addition to this, the use of microorganisms provides the basis for indicating the type of antibacterial substances or the extracts proved useful for specific pathogens. In the last few years, listeriosis has undergone a transformation from an infectious disease of limited importance to one of the most topical food-borne infections and a source of major concern for health authorities and the food industry alike. In the same period of time, the work of various groups has made the causal agent of this disease, *L. monocytogenes*, one of the best-characterized intracellular parasites at both molecular and cellular levels. Historically, various microorganism based substances have been reported to have antilisterial properties[19]. Also, the renewal of interest in food or pharmaceutical industry for effective, safe natural products means that quantitative data

**Table 3. Effect of ethyl acetate extract of a marine isolate of *Aspergillus* sp. on viability of the tested bacterial pathogen**

| Microorganism | Viable count (Log CFU $ml^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Time (min) | | | | | |
| | **0** | **40** | **60** | **80** | **100** | **140** |
| *Listeria monocytogenes* ATCC 19166 (Treatment)[a] | 7.0 | 0.5 | 0.2 | 0 | 0 | 0 |
| *Listeria monocytogenes* ATCC 19166 (CT)[b] | 7.0 | 7.1 | 7.2 | 7.3 | 7.4 | 7.6 |

[a] Treatment at MIC concentration of ethyl acetate extract (125 μg $ml^{-1}$); [b] Control without treatment.

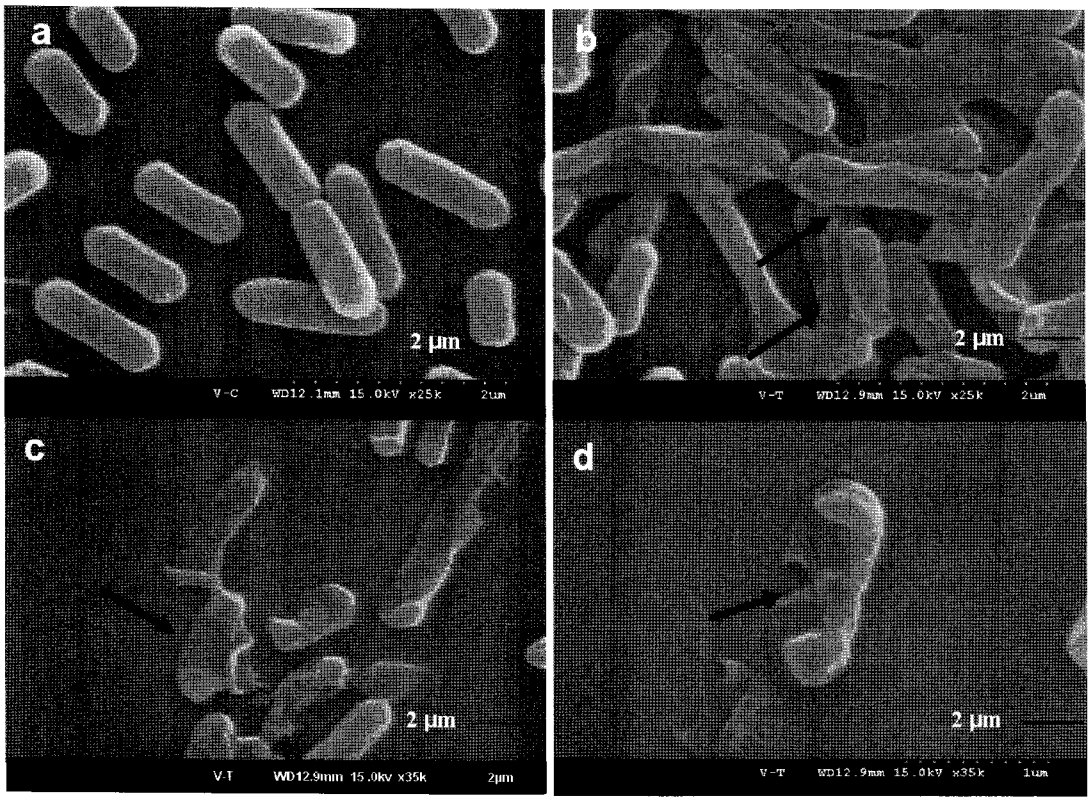


**Fig. 1. Scanning electron micrographs of *Listeria monocytogenes* ATCC 19166 treated with ethyl acetate extract (125 μg $ml^{-1}$) of a marine isolate of *Aspergillus* sp. (a) Control (b) disruption and abnormal cell formation; (c) lysis of membrane integrity (d) wrinkled abnormalities and cleft formation.**

based on naturally occurring antibacterial substances including extracts are required to control serious human pathogenic disorders.

Our study revealed significant results of antilisterial effect of EtOAc extract of *Aspergillus* sp. against the tested strains of *L. monocytogenes*. These findings are in agreement with previous observation of other researchers who observed similar results of antilisterial effect using organic extracts[20]. These results suggest the availability of various organic extracts from different origins for trials in controlling such infectious *Listeria* organisms. Therefore, as a result, work on alternative approaches to control such pathogens is important.

Overall, based on the above results, it can be estimated that EtOAc extract of *Aspergillus* sp. has strong antilisterial effect against *L. monocytogenes* strains, therefore, EtOAc extract of *Aspergillus* sp. can be used as a potential antimicrobial agent to control *L. monocytogenes* as well as other pathogenic microorganisms. The development of natural antibacterial agents would also help to decrease the negative impact of synthetic agents, such as residues, resistance and environmental pollution. In this respect, natural bactericides may be effective, selective, biodegradable, and less toxic to environment. However, little has known about the mechanisms used by pathogenic *Listeria* spp. to penetrate the organism, to cross the host's physiological barriers, and to interact with the target cells in which they proliferate, causing disease.

In conclusion, the primary significance of this study is an observation that EtOAc extract could effectively inhibit the growth of *L. monocytogens* strains, *Listeria* infections that have caused patients to seek medical attention. To the best of our knowledge, this is the first study on the effects of EtOAc extract of a marine derived fungus against a human pathogen *L. monocytogenes*. Thus it can be estimated that the EtOAc extract derived from *Aspergillus* sp. may potentially contribute in the fields of food, medical microbiology and environmental industries as the supplement to cure pathogenic bacteria in future as the fast and reliable alternative. Further study is required to apply isolation techniques to identify the bioactive components present in the ethyl acetate extract of *Aspergillus* sp. and their action of mechanism for such antilisterial effect.

## REFERENCES


1. Montville, T. J., Winkowski, K., and Ludescher, R. D. (1995) Models and mechanisms for bacteriocin action and application. *Int. Dairy* J. 5, 797-814.
2. Farber, J. M., and Peterkin, P. I. (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55, 476-511.
3. Broseta, S. M., Diot, A., Bastian, S., Riviere, J., and Cerf, O. (2003) Estimation of low bacterial concentration: *Listeria monocytogenes* in raw milk. *Int. J. Food Microbiol.* 80, 1-15.
4. Dalton, C. B., Austin, C. C., Sobel, J. P., Hayes, S., Bibb, W. F., Graves, L. M., Swaminathan, B., Proctor, M. E., and Griffin, P. M. (1997) An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. *N. Engl. J. Med.* 336, 100-105.
5. Salamina, G. E., Donne, D., Nicolini, A., Poda, G., Cesarone, D., Bucci, M., Fini, R., Maldini, M., Schuchat, A., Swaminathan, B., Bibb, W., Rocourt, J., Binkin, N., and Salmaso, S. (1996) A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. *Epidemiol. Infect.* 117, 429-436.
6. Fenlon, D. R. (1999) *Listeria monocytogenes* in the natural environment. In: Ryser ET, Marth EH, eds. *Listeria*, listeriosis, and food safety, 2nd eds. New York: Marcel Dekker Inc.
7. Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G.., Goebel, W., Gonzalez-Zorn, B., Wehland, J., and Kreft, J. (2001) *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14, 584-640.
8. Bille, J. (1990) Epidemiology of human listeriosis in Europe, with special reference to the Swiss outbreak. In: Miller AJ, Smith JL, Somkuti GA, eds. Foodborne listeriosis. New York: Elsevier.
9. Schlech, W. F. III (2000) Foodborne listeriosis. *Clin. Infect. Dis.* 31, 770-775.
10. Shen, H. C., Tato, M., and Fan, X. (1998) *Listeria monocytogenes* as a probe to study cell-mediated immunity. *Curr. Opin. Immunol.* 10, 450-458.
11. Martinez, R. C. R., and De Martinis, E. C. P. (2005) Antilisterial activity of a crude preparation of *Lactobacillus sakei* 1 bacteriocin and its lack of influence on *Listeria monocytogenes* haemolytic activity. *Food Control* 16, 429-433.

12. Faulkner, D. J. (2001) Marine natural products. *Nat. Prod. Rep.* 18, 1-49.
13. Bajpai, V. K., and Kang, S. C. (2008) The antidermatophytic potential of the marine isolate of *Aspergillus* sp. collected from South Coast of Korea. *K. J. Environ. Agric.* 27(1), 80-85.
14. Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., and Yolke, R. H. (1995) Manual of Clinical Microbiology, Vol. 6, eds. Washington: ASM Press.
15. Chandrasekaran, M., and Venkatesalu, V. (2004) Antibacterial and antifungal activity of *Syzygium jambolanum* seeds. *J. Ethnopharmacol.* 91, 105-108.
16. Shin, S. Y., Bajpai, V. K., Kim, H. R., and Kang, S. C. (2007) Antibacterial activity of eicosapentaenoic acid (EPA) against foodborne and food spoilage microorganisms. *LWT* 40, 1515-1519.
17. Kockro, R. A., Hampl, J. A., Jansen, B., Peters, G.., Scheihing, M., and Giacomelli, R. (2000) Use of scanning electron microscopy to investigate the prophylactic efficacy of rifampin-impregnated CSF shunt catheters. *J. Med. Microbiol.* 49, 441-450.
18. Koyama, S., Yamaguchi, Y., Tanaka, S., and Motoyashiya, J. (1997) A new substance (yoshixol) with an interesting antibiotic mechanism from wood oil of Japanese traditional tree (kiso-hinoki), *Chamaecyparis obtuse. Gen. Pharmacol.* 28, 797-804.
19. Song, H. J., and Richard, J. (1997) Antilisterial activity of three bacteriocins used at sub minimal inhibitory concentrations and cross-resistance of the survivors. *Int. J. Food Microbiol.* 36, 155-161.
20. Roxanne, J. O., and Enzo, A. P. (2005) Antilisterial activity of ethanolic extracts of medicinal plants, *Eremophila alternifolia* and *Eremophila duttonii*, in food homogenates and milk. *Food Control* 18, 387-390.