Korean Journal of Environmental Agriculture

Korean J Environ Agric (2011)
Vol. 30, No. 4, pp. 459-465   http://dx.doi.org/10.5338/KJEA.2011.30.4.459

Online ISSN: 2233-4173
Print ISSN: 1225-3537

Antibacterial Effects of Extracts of *Thuja Orientalis* cv *Aurea* Nana Cones against Food-spoilage and Food-borne Pathogens

Xiao Nan Yang,[1] Cher-Won Hwang,[2] Gi-Seok Kwon[3] and Sun Chul Kang[1*]

[1]Department of Biotechnology, Daegu University, Gyeongsan, Gyeongbuk 712-714, Republic of Korea

[2]School of Global Leadership, Handong Global University, Pohang, 791-708, Republic of Korea

[3]Dept. of Bioresource Sciences, Andong National University, Andong 760-749, Republic of Korea

Abstract

BACKGROUND: Nowadays, Chemical antiseptics have become great problems for health and environmental, so that developing of new substitutes for chemical antiseptics is more and more important. Natural product is a kind of environment-friendly additive that could be used as antiseptic in food industry. *Thuja orientalis* cv *Aurea* Nana is a gymnospermous plant of the family Cupressaceae, native to northwestern China and widely naturalised elsewhere in Korea and Japan. This study was aimed to investigate the antibacterial potential of various organic extracts from *T. orientalis* cones against some food-borne and food-spoilage bacteria.

METHODS AND RESULTS: Hexane extract (HE), chloroform extract (CE), ethyl acetate extract (EAE) and methanol extract (ME) were obtained from female cones of *T. orientalis*. The antibacterial activities of various extracts were tested by standard agar diffusion and minimum inhibitory concentrations (MICs) against five gram-positive and six gram-negative bacteria. Cell viability and morphology change of *L. monocytogenes* ATCC 10943 treated with hexane extract were also observed. The various extracts displayed remarkable antibacterial effects against all the gram-positive bacteria but did not show any effect against the gram-negative bacteria. Hexane extract has the highest inhibitory effect on cell viability of *L. monocytogenes* ATCC 10943. SEM observation also demonstrated the damaging effect of the hexane extract on the morphology of *L. monocytogenes* ATCC 10943 at the minimum inhibitory concentration.

CONCLUSION(s): The tested gram-positive bacteria were significantly inhibited by organic extracts of *T. orientalis* cone. Hexane extract was the most potent against *Listeria monocytogenes* ATCC 10943, as evidenced by the lowest MIC level and the complete inhibition of cell viability within shortest exposure time, along with SEM observation.

Key Words: Antibacterial activity, Food-borne and Food-spoilage pathogens, *Thuja orientalis* cv *Aurea* Nana

*Prof. Sun Chul Kang Corresponding author,
Department of Biotechnology, College of Engineering, Daegu University, Kyoungsan, Kyoungbook 712-714, Republic of Korea
Phone: +82-53-850-6553; Fax: +82-53-850-6559;
E-mail: sckang@daegu.ac.kr; Phone: +82-53-850-6553;
Fax: +82-53-850-6559

Introduction

Food-borne diseases encompass a wide spectrum of illnesses and are a growing public health problem worldwide. In industrialized countries, 30% of populations are affected by food-borne diseases annually, and the problem is likely to be even more widespread in developing countries. In spite of modern improvements in slaughter hygiene and food production techniques, food safety is an increasingly important public health issue

(WHO, 2002). Mostly, the food industries are using chemical preservatives to prevent the growth of food-borne and food-spoiling microbes. Some natural products have also been used as antiseptics to reduce the amount of the chemicals added to food or to substitute them. Natural plant extracts are of growing interest to both of food industry and researchers because their antibacterial, antifungal, and antioxidant properties provide a great potential as an alternative to the chemical food preservatives (Deba *et al.*, 2008).

Thuja orientalis (=*Platycladus orientalis* (L.)) is a gymnospermous plant of the family Cupressaceae, which is native to northwestern China and widely distributes in Northeast Asian countries including Korea and Japan. *T. orientalis* cv *Aurea* Nana is a dwarf plant with yellow-green leaves that compactly branch, which widely used as an ornamental plant in South Korea. It has been reported that the leaves of *T. orientalis* were used in the treatment of various inflammatory diseases (Kim *et al.*, 2010). In Chinese herbal medicine, the leaves have been used for treatments of gout, rheumatism, diarrhea and chronic tracheitis (Zhu *et al.*, 2004). The plant also exhibits anti-plasmodial (Asili *et al.*, 2004), fungi toxic (Guleria *et al.*, 2008) and molluscicidal (Singh and Singh, 2009) effects, and improves impaired memory acquisition (Nishiyama *et al.*, 1995). There are many reports on such phytochemical and biological studies of this plant leaves all over the world. However, other parts of this plant have not been adequately studied for their medicinal or industrial use. Therefore, this study was carried out to investigate *in vitro* antibacterial activity of seed cone extracts of *T. orientalis*.

Materials and methods

Plant material

5 kg of female cones of *T. orientalis* were collected from local area of Kyungsan, Kyungpook, South Korea in October, 2010. The collected plant was identified to species level by morphological features. A voucher specimen (DU-TO396) was deposited at Daegu University for further reference. The cones were washed, dried and ground.

Samples extraction

The ground cones (150 g) of *T. orientalis* were extracted with 500 ml of four different organic solvents including hexane, chloroform, ethyl acetate and methanol for 7

days at room temperature, respectively. And then, the solvents were evaporated by vacuum rotary evaporator (EYELA N1000, Japan) to get the hexane extract (HE), chloroform extract (CE), ethyl acetate extract (EAE) and methanol extract (ME). The extracts were maintained at 4°C until further use. Solvents (analytical grade) for extraction were obtained from a commercial source (SAMCHUN PURE CHEMICAL CO., LTD, Korea).

Test microorganisms (food-spoilage and food-borne pathogens)

Eleven food-spoilage and food-borne pathogens used in this study include five gram-positive bacteria (*Bacillus cereus* KCTC 14042, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 10943, *Staphylococcus aureus* Wild Type, *Staphylococcus aureus* ATCC 6538) and six gram-negative bacteria (*Escherichia coli* 0157 KCTC 14034, *Escherichia coli* ATCC 10536, *Escherichia coli* ATCC 8739, *Salmonella enteritidis* KCTC 12243, *Salmonella typhimurium* Wild Type, *Pseudomonas aeruginose* ATCC 15522). Fresh cultures were prepared by transferring a loop of cells from each stock culture to a new tube containing Luria-Bertani (LB) broth medium. The cultures were grown at 37°C for 24 h and maintained on LB agar medium at 4°C.

Assay for antibacterial potential

Standard agar diffusion method was used for antibacterial assay (Murray et al., 1995). Organic extracts were dissolved in DMSO. Fresh bacterial cultures were prepared by transferring a loop of cells from the stock culture to a flask containing LB medium, and incubated at 37°C for 24 h. The cultures were diluted with LB broth to achieve an optical density of 10^7 CFU/ml for the test organisms at 600 nm by UV/Vis Spectrophotometer Optizen 2120 UV. 0.1 ml of each standardized bacterial inoculum (10^7 CFU/ml) was poured into Petri plates containing 20 ml of LB agar medium, uniformly spread, and allowed to dry for 5 min. Sterile filter paper discs (6 mm diameter, Waterman No.1) were impregnated with 1 mg/disc of HE, CE, EAE and ME, respectively, and placed on the inoculated agar plates. After the plates were kept at room temperature for 30 min to allow the extracts to diffuse into the agar, they were incubated at 37°C for 24 h. Antibacterial activity of the extracts was evaluated by measuring the diameter of inhibition zone developing around the discs.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of extracts were tested by the method described by Chandrasekaran and Venkatesalu (2004). Each extract was incorporated into LB broth medium in a tube and adjusted to a final concentration in the range of 0 to 1500 μg/ml. 10 μl of standardized suspension of each test organism (10^7 CFU/ml) was transferred to the tubes, and incubated at 37°C for 24 h. MIC was determined as the lowest concentration (μg/ml) of each extract where no visible growth of test organisms occur.

Effect of hexane extract on cell viability of *Listeria monocytogenes*

Hexane extract at the MIC in 10 ml of LB broth was added into the tubes containing bacterial suspension (approximately 10^7 CFU/ml) of *L. monocytogenes* ATCC in LB broth medium. After incubation at 37°C, 10 μl of samples for viable cell counts were removed at 30-min intervals up to 240 min. Each sample was diluted appropriately with sterile water, and 50 μl of the diluted suspension was spread on the surface of LB agar. Colonies that formed on each plate were counted after 24 h of incubation at 37°C. The control which inoculated without hexane extract was treated at same experimental condition as mentioned above.

Scanning electron microscopy analysis

The method of sample preparation for scanning electron microscopy (SEM) was modified from that of Kockro *et, al* (2000). Bacterial cells of *L. monocytogenes* ATCC 10943 which were treated with and without the MIC level of hexane extract for 4 h were washed three times using 50 mM phosphate buffer solution (PBS, pH 7.3), and centrifuged at 4000 g. The supernatant was removed and the centrifuged cells were suspended in a new PBS. A thin smear of the suspension was coated on a glass slide (4 mm^2) and fixed for 3 h in 2.5% (v/v) glutaraldehyde (Electron Microscopy Science, Washington, USA). After the fixation, the bacterial cells on each glass slide were rinsed, dehydrated using a series of graded ethanol (concentrations ranging from 50% to 100%), and dried with CO_2. The dried cells were coated with gold in a sputter coater (Hitachi, Japan). Samples were observed under a Scanning electron microscope (Hitachi-S4300, Japan).

Table 1. Diameter of inhibition zones (mm) produced by cone extracts of *T. orientalis* on the test pathogens.

Microorganism	Diameter of inhibition zones [A] (mm)			
	HE	CE	EAE	ME
Bacillus cereus KCTC 14042	22.8 ± 0.9 [a]	19.7 ± 0.7 [a]	17.8 ± 0.7 [a]	26.2 ± 0.8 [a]
Bacillus subtilis ATCC 6633	10.2 ± 0.4 [e]	12.2 ± 0.8 [d]	12.8 ± 0.4 [c]	13.2 ± 0.5 [d]
Listeria monocytogenes ATCC 10943	11.4 ± 0.6 [d]	12.7 ± 0.8 [d]	18.5 ± 0.8 [a]	16.3 ± 0.6 [c]
Staphylococcus aureus Wild Type	17.5 ± 0.8 [b]	15.0 ± 0.5 [c]	15.9 ± 0.8 [b]	16.0 ± 0.6 [c]
Staphylococcus aureus ATCC 6538	15.1 ± 0.4 [c]	16.3 ± 0.3 [b]	17.2 ± 0.7 [a]	18.0 ± 0.4 [b]
Escherichia coli 0157 KCTC 14034	nd [B]	nd	nd	nd
Escherichia coli ATCC 10536	nd	nd	nd	nd
Escherichia coli ATCC 8739	nd	nd	nd	nd
Salmonella enteritidis KCTC 12243	nd	nd	nd	nd
Salmonella typhimurium Wild Type	nd	nd	nd	nd
Pseudomonas aeruginosa ATCC 15522	nd	nd	nd	nd

[A] Mean ± SD. Results are based on triplicate tests. Different letters next to the values indicate significant differences at $p < 0.05$. (Tested concentration of extracts: 1 mg/disc). HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract, ME: methanol extract.
[B] nd, no inhibition detected.

Table 2. Minimum inhibitory concentrations of cone extracts of *T. orientalis* against the growth of food-spoilage and food-borne pathogens.

Microorganism	MICs [A]			
	HE	CE	EAE	ME
Bacillus cereus KCTC 14042	10	75	500	500
Bacillus subtilis ATCC 6633	10	75	75	100
Listeria monocytogenes ATCC 10943	25	100	750	500
Staphylococcus aureus Wild Type	50	750	500	500
Staphylococcus aureus ATCC 6538	10	750	750	500
Escherichia coli 0157 KCTC 14034	nd [B]	nd	nd	nd
Escherichia coli ATCC 10536	nd	nd	nd	nd
Escherichia coli ATCC 8739	nd	nd	nd	nd
Salmonella enteritidis KCTC 12243	nd	nd	nd	nd
Salmonella typhimurium Wild Type	nd	nd	nd	nd
Pseudomonas aeruginosa ATCC 15522	nd	nd	nd	nd

[A] MICs, Minimum inhibitory concentrations (values in μg/ml). HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract, ME: methanol extract.
[B] nd, no inhibition detected.

Results

In vitro antibacterial activity

In vitro antibacterial activities of *T. orientalis* extracts were determined by the presence/absence and the diameter of inhibition zone (Table 1). All extracts were found to have antibacterial effects against all gram-positive bacteria tested, but none of the gram-negative bacteria. The diameters of inhibition zones of HE, CE, EAE and ME against the gram-positive bacteria were in the range of 10.2-22.8 mm, 12.2-19.7 mm, 12.8-18.5 mm and 13.2-26.2 mm, respectively. For all of the extracts, the largest inhibition zone was developed against *B. cereus* KCTC 14042 (17.8-26.2 mm). Among all the extract tested, ME exhibited the highest antibacterial activities against *B. cereus* KCTC 14042, *B. subtilis* ATCC 6633 and *S. aureus* ATCC 6538. Its inhibitory effect was also high against *L. monocytogenes* ATCC 10943 and *S. aureus* Wild Type.

Minimum inhibitory concentrations

The minimum inhibitory concentrations of various extracts against the gram-positive bacteria tested were in the range of 10-750 μg/ml (Table 2). The HE showed the most potent inhibitory capacity with the MICs in the range of 10-50 μg/ml. The CE was more potent to *B. cereus* KCTC 14042, *B. subtilis* ATCC 6633 and *L. monocytogenes* ATCC 10943 compared to EAE and ME. However, the inhibitory effects against
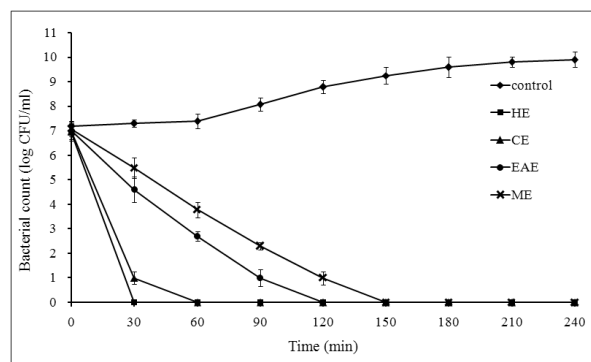

Fig. 1. Effects of extracts of *T. orientalis* cones (at MIC levels) on the viability of the *L. monocytogenes* ATCC 10943. Control cells were not treated with the extracts. Bars indicate standard errors. HE: hexane extract, CE: chloroform extract, EAE: ethyl acetate extract, ME: methanol extract.

S. aureus Wild Type and *S. aureus* ATCC 6538 were stronger than vice versa when treated with EAE and ME. The EAE and ME also showed potential inhibitory effects against all the test gram-positive bacteria with the MICs in the range of 75-750 μg/ml and 100-500 μg/ml, respectively. None of the extracts inhibited the growth of the five gram-negative bacteria tested.

Effects of extracts on cell viability of bacterium

Reduced viability of *L. monocytogenes* ATCC 10943 was observed at MIC of all extracts tested (Fig. 1). After 150 min exposure, all the extracts revealed complete inhibition of CFU number against *L.*
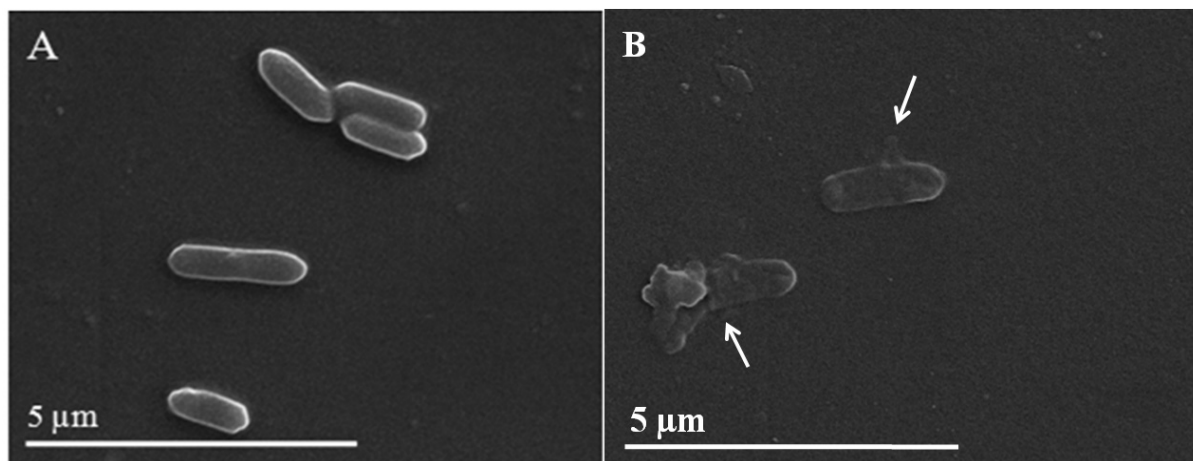
Fig. 2. Effect of hexane extract of *T. orientalis* cones on the morphology of *L. monocytogenes* ATCC 10943. A: cells without treatment showing a regular, smooth surface; B: cells lysis (arrows) caused by hexane extract inoculation at the MIC level (25 µg/ml).

monocytogenes ATCC 10943. All of the extracts but ME induced 1-fold inhibition of cell viability within 60 min. A sharp decrease in the CFU number was observed within 30 min when treated with CE. *L. monocytogenes* ATCC 10943 was found to be the most sensitive to the HE, as evidenced by the complete inhibition of cell viability within 30 min of exposure.

Scanning electron microscopy

HE was found to alter cell morphology of *L. monocytogenes* ATCC 10943 by scanning electron microscopy as compared to control group (Fig. 2). Control cells showed a regular, smooth surface as shown in Fig. 2A. In contrast, inoculation with HE at the MIC level (25 µg/ml) appeared to have a severe detrimental effect on cell structures of *L. monocytogenes* ATCC 10943, resulting in cell lysis, as shown in Fig. 2B.

Discussion

Plant extracts have been used for various purposes for thousands of years (Jones, 1996). Historically, many plant extracts have been used as topical antiseptics, or have been reported to have antimicrobial properties (Hoffman, 1987; Lawless, 1995). Some components of plant extracts are also used as food flavorant or antibacterial agent (Helander *et al.*, 1998). Because of the potential as a source of novel antimicrobial compounds, there has been the need for scientific researches on plants that have been used in traditional medicine (Hammer *et al.*, 1999).

In the present study, the results of the minimum inhibitory concentrations showed that extracts of *T. orientalis* cone exhibited potent activities against some food-spoilage and food-borne pathogenic bacteria such as *B. cereus* KCTC 14042, *B. subtilis* ATCC 6633, *L. monocytogenes* ATCC 10943, *S. aureus* Wild Type, and *S. aureus* ATCC 6538. The HE exhibited remarkable inhibitory activities against all gram-positive bacteria tested, as evidenced by the lowest MIC values and the complete inhibition of cell viability of *L. monocytogenes* ATCC 10943 after a 30-min exposure. This might be the result of the action of non-polar compounds such as terpenoids which also exist in essential oil of *T. orientalis* cones (Hassanzadeh *et al*, 2001). CE, EAE and ME also showed considerable inhibitory activities against all the gram-positive bacteria. The abundant phenolic compounds might contribute the antibacterial activities of these extracts (Lu *et al.*, 2006). Phenolic compounds are often involved in the degradation of cell walls and cell membranes, as well as the disruption of membrane functions such as electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity (Nychas, 1995). Thus, some phenolic compounds in the above extracts may have contributed to the inhibition of the growth and viability of the test microorganisms.

The gram-negative bacteria were more resistant to the action of the organic extracts then the gram-positive bacteria tested. Their high resistance is likely due to the outer membrane surrounding their cell wall, which restricts permeation of hydrophobic compounds through the lipopolysaccharide layer (Vaara, 1992).

The HE produced smaller inhibition zones than the other extracts with higher polarity in most cases.

However, its MIC values were much lower than those of other extracts. This might suggest that some compounds present in the HE could not readily diffuse throughout the agar because of its hydrophobicity. On the other hand, the compounds present in the other extracts are more hydrophilic, which seem to diffuse rapidly into agar. All the compounds, however, appeared to distribute evenly in broth media.

The HE was found to have stronger inhibitory effects on the growth and viability of test microorganisms than the other types of extracts. The hydrophobic components present in HE are likely to damage the lipid membranes of bacterial cells and mitochondria, thus increasing the permeability of the affected cells (Sikkema *et al.*, 1994).

Conclusions

The present study demonstrated that organic extracts of *T. orientalis* cones have significant inhibitory effects against some gram-positive bacteria, which are associated with food-spoilage and food-borne diseases. These results provide evidence that cone extracts of *T. orientalis* has the potential as a useful alternative to chemical food preservatives for controlling the growth of food-spoilage and food-borne pathogens.

Acknowledgments

References

Asili, J., Lambert, M., Ziegler, H.L., Staerk, D., Sairafianpour, M., Witt, M., Asghari, G., Ibrahimi, I.S., and Jaroszewski, J.W., 2004. Labdanes and isopimaranes from *Platycladus orientalis* and their effects on erythrocyte membrane and on *Plasmodium falciparum* growth in the erythrocyte host cells,. *J. Nat. Prod.* 67, 631-637.

Chandrasekaran, M., and Venkatesalu, V., 2004. Antibacterial and antifungal activity of *Syzygium jambolanum* seeds,. *J. Ethnopharmacol.* 91, 105-108.

Deba, F., Xuan, T.D., Yasuda, M., and Tawata, S., 2008. Chemical composition and antioxidant, antibacterial and antifungal activities of the essential oils from *Bidens pilosa* Linn. var. *Radiata., Food Control.* 19, 346-352.

Guleria, S., Kumar, A., and Tiku, A.K., 2008. Chemical composition and fungitoxic activity of essential oil of *Thuja orientalis* L. grown in the north-western Himalaya. *Z. Naturforsch.*, 63c, 211-214.

Hammer, K.A., Carsonl, C.F., and Riley, T.V., 1999. Antimicrobial activity of essential oils and other plant extracts,. *J. Appl. Microbiol.* 86, 985-990.

Hassanzadeh, M.K., Rahiimizadeh, M., Fazly Bazzaz, B.S., Emami, S.A., and Assili, J., 2001. Chemical and antimicrobial studies of *Platycladus orientalis* essential oils,. *Pharm. Biol.* 39, 388-390.

Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., and Wright, A.von., 1998. Characterization of the action of selected essential oil components on gram-negative bacteria,. *J. Agric. Food Chem.* 46, 3590-3595.

Hoffman, D.L., 1987. The Herb User's Guide. Wellingb., UK: Thorsons Publishing Group.

Jones, F.A., 1996. Herbs-useful plants. Their role in history and today,. *Eur. J. Gastroenterol. Hepatol.* 8, 1227-1231.

Kim, J.Y., Kim, H.J., Kim, S.M., Park, K.R., Jang, H.J., Lee, E.H., Jung, S.H., and Ahn, K.S., 2010. Methylene chloride fraction of the leaves of *Thuja orientalis* inhibits *in vitro* inflammatory biomarkers by blocking NF-κB and p38 MAPK signaling and protects mice from lethal endotoxemia,. *J. Ethnopharmacol.* 133, 687-695.

Kockro, R.A., Hampl, J.A., Jansen, B., Peters, G., Scheihing, M., and Giacomelli, R, 2000. Use of scanning electron microscophy to investigate the prophylactic efficacy of rifampin-impregnated CSF shunt catheters,. *J. Med. Microbiol.* 49, 441-450.

Lawless, J., 1995. The Illustrated Encyclopedia of Essential Oils. Shaftesbury, UK: Element Books Ltd.

Lu, Y.H., Liu, Z.Y., Wang, Z.T., and Wei, D.Z., 2006. Quality evaluation of *Platycladus orientalis* (L.) Franco through simultaneous determination of four bioactive flavonoids by high-performance liquid chromatography,. *J. Pharm. Biomed. Anal.* 41, 1186-1190.

Murray, P.P., Baron, E.J., Pfaller, M.A., Tenove, F.C., and Yolke, R.H., 1995. Manual of Clinical Microbiology, ASM, Washington.

Nishiyama, N., Chu, P.J., and Saito, H., 1995. Beneficial effects of biota, a traditional Chinese herbal medicine on learning impairment induced by basal forebrain-lesion

in mice,. *Biol. Pharm. Bull.* 18, 1513-1517.

Nychas, G.J.E., 1995. Natural Antimicrobials from Plants, G.W. Gould, Editor, *New methods of food preservation*, Blackie Academic Professional, London.

Sikkema, J., De Bont, J.A.M., and Poolman, B., 1994. Interactions of cyclic hydrocarbons with biological membranes,. *J. Biol. Chem.* 269 (11), 8022-8028.

Singh, A., and Singh, V.K., 2009. Molluscicidal activity of *Saraca asoca* and *Thuja orientalis* against the fresh water snail *Lymnaea acuminate.*, *Vet. Parasitol.* 164, 206-210.

Vaara, M., 1992. Agents that increase the permeability of the outer membrane,. *Microbiol. Rev.* 56(3), 395-441.

WHO, 2002. Food Safety and Foodborne Illness. World Health Organization Fact sheet 237, Geneva.

Zhu, J.X., Wang, Y., Kong, L.D., Yang, C., and Zhang, X., 2004. Effects of *Biota orientalis* extract and its flavonoid constituents, quercetin and rutin on serum uric acid levels in oxonate-induced mice and xanthine dehydrogenase and xanthine oxidase activities in mouse liver,. *J. Ethnopharmacol.* 93, 133-140.