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Synergistic antimicrobial efficacy of mesoporous ZnO loaded with 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate isolated from the *Moringa oleifera* seed

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The antimicrobial activities of isolated compounds from seed extracts of *Moringa oleifera* and synergistic antimicrobial efficacy through hybridized complex of organic-inorganic composite materials were studied. The two main components of the *Moringa oleifera* seed were isolated and determined to be niazimicin and 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate (RBI). The antimicrobial activity of the separated compounds of the *Moringa oleifera* seed were tested in vitro against 3 bacterial species and 2 fungal species by the paper disc diffusion assay and broth dilution methods. Both compounds showed antimicrobial activity against tested species and RBI was more effective than niazimicin. The MIC of RBI on *S. aureus*, *E. coli*, *P. aeruginosa, C. albicans,* and *A. niger* was 0.005%, 0.1%, 0.5%, 0.5%, and 0.5%, respectively, while the MIC of niazimicin on *S. aureus* was 0.1%. Next, we investigated the combined antimicrobial action of mesoporous ZnO and RBI by incorporating the compound within the pore of mesoporous ZnO. The MIC of mesoporous ZnO with RBI on *S. aureus*, *E. coli*, *P. aeruginosa, C. albicans,* and *A. niger* was 0.001%, 0.01%, 0.5%, 0.1%, and 0.1%, respectively. A synergistic effect of RBI with mesoporous ZnO was shown. From these results, the mesoporous ZnO could act as a reservoir for RBI and mesoporous ZnO with RBI could be used for cosmetic preservatives.

Key words: antimicrobial activity; 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate; mesoporous ZnO; *Moringa oleifera*; niazimicin

Introduction

In cosmetics, various synthetic preservatives have been used to prevent contamination that results from microorganism proliferation. However, some synthetic preservatives are reported to cause skin irritation, erythema, contact allergy, contact sensitization, and contact dermatitis (Sasseville, 2004). For example, parabens are widely used preservatives, which are reported to increase the risk of breast cancer and influence the development of malignant melanoma (Golden et al., 2005). Moreover, formaldehyde-releasing preservatives such as imidazolidinyl urea and diazolidinyl urea are thought to cause a skin reaction in sensitive individuals, and allergies to isothiazolinones have been reported in many publications (Reinhard et al., 2001; Varvaresou et al., 2009).

Thus researchers are trying to find safe and effective alternative preservatives to resolve the safety problems of synthetic preservatives. Many studies have been done on materials from various plants. Extracts of plants such as garlic, pine leaf, mugwort, and medicinal herbs were found to have antimicrobial activity (Zoghbi, Maria das Gracas B et al., 1984).

Among them, *Moringa oleifera* is a perennial legume, widely distributed in India, Thailand, the Philippines, Africa, and Asia. *M. oleifera* has been used in traditional medicines (Jahn and Dirar, 1979) to treat heart complications, eye diseases, tumors, earaches, and dyspepsia. Furthermore, it is considered to be useful in treating burns, cholera, epilepsy, and retention of urine (Eilert et al., 1981). The pharmacological activity of *M. oleifera* has been studied at length,

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including the antimicrobial activity of the seeds, as well as the spasmolytic, anti-inflammatory properties of the leaves, fruit, bark, and roots (Caceres et al., 1991). According to recently reported studies, a great deal of research has been carried out on its antimicrobial (Chuang et al., 2007), anti-cancer (Park et al., 2011) and anti-inflammatory activity (Sreelatha et al., 2011).

The nanostructured particles of mesoporous were demonstrated to act as an effective carrier for the sustained delivery of antimicrobial agents with an enhanced inhibitory activity (Carmona et al., 2012; Morones et al., 2005; Park et al., 2011; Ruparelia et al., 2008). Nanoparticles themselves also can exert antimicrobial activity through disrupting cell membranes directly (Liu et al., 2009).

In this study, we isolated two main components of the seed extract of *M. oleifera* which showed antimicrobial activity. The antimicrobial activity of niazimicin and RBI were tested on bacteria and fungi. We also tested the synergistic antimicrobial activity of RBI with mesoporous ZnO.

Materials and Methods

Reagent, materials, plant. ^{1}H and ^{13}C-NMR spectra were determined using a Varian UNMR-400 spectrophotometer operating at 400 MHz for protons and 100 MHz for carbon, respectively. All of the chemicals were purchased from Sigma Aldrich Chemical Company (St. Louis, MO). Distilled water was prepared with a Milli-Q water purification system from Millipore (Molsheim, France). For the microbial tests, tryptic soy agar (Difco, Detroit, MI), Sabouraud dextrose agar (Difco) and nutrient broth (Difco) were used. *M. oleifera* seeds were purchased from Kyeong-Dong Market in Seoul, Korea.

Extraction and isolation. The *M. oleifera* seeds were dried in the sun for 3 days and ground into a fine powder. The *M. oleifera* seed extract (100 g) was then dissolved in 70% (v/v) ethanol and filtered through a 1.2 μm membrane filter. To get 8.3 g of *M. oleifera* seed extract from the filtrate, we tried to do vacuum evaporation with reduced pressure under 50°C.

Alternatively, hexane was used to obtain a certain *M. oleifera* seed extract. Polar/non-polar fractionation was used as the separation method to remove oil. After standing, the non-polar layer was removed once the polar (water)/ non-polar (*n*-hexane) layer had separated.

The Ethyl Acetate (EA) layer was obtained by leaving the two phases to separate after the same amount had been mixed in the aqueous layer and EA. To get 4.5 g of *M. oleifera* seed extract from the EA layer, we tried to do vacuum evaporation with reduced pressure under 50°C

M. oleifera seed extracts were dissolved in methanol. This solution was fractionated by preparative medium pressure liquid chromatography (MPLC). The chromatographic separation was performed on a CombiFlash Rf 200 (Teledyne Isco, Lincoln, NE) Preparative Chromatography system. The preparative medium pressure liquid chromatography was performed on a Reveleris C_{18} reverse phase cartridge (80 g, Grace, Columbia, MD). The mobile phase used distilled water (solvent A) and CH_3CN (solvent B). A gradient elution program of 0−25 min, 5% B; 25−65 min, 5−25% B, 65−140 min, 25−30% B; 140−180 min, 30−100% B was used. The flow rate was 15 ml/min, and the detection wavelength was 254 nm. We separated two compounds from the 1 g extract as follows: niazimicin (50 mg, tR 95 min), RBI (300 mg, tR 123 min). The two compounds were confirmed by analysis of the ^{1}H and ^{13}C-NMR spectral data (Cheenpracha et al., 2010; Eilert et al., 1981).

Test organism and medium. The microorganisms below were used in the antimicrobial test because they are suggested as challenge microorganisms in the Cosmetic, Toiletry, and Fragrance Association (CTFA) Microbiology Guideline: *Staphylococcus aureus* (*S. aureus*) (ATCC6538), *Escherichia coli* (*E. coli*) (ATCC8542), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC9027), *Candida albicans* (*C. albicans*) (ATCC10231), and *Aspergillus niger* (*A. niger*) (ATCC9642). Bacteria were incubated in tryptic soy agar medium, and the fungi were incubated in Sabouraud dextrose agar medium.

Antimicrobial screening. The paper disc diffusion method was used to test the antimicrobial activity of the separated compounds (Loo et al., 1945). An inoculum of bacteria was cultured on tryptic soy agar (Difco) at 37°C for 24 h, and inocula of *C. albicans* and *A. niger* were grown on Sabouraud dextrose agar (Difco) at 25°C for 48 h and 5 days, respectively. Inocula of fungi were prepared by harvesting in 0.85% saline water containing 0.05% Tween-80 (Sigma), adjusting the number of cells to approximately 10^5 spores/ml, and bacteria to 10^6 CFU/ml. Microorganisms were loaded on solid agar plates, and the disc loaded with each compound was put on a solid agar plate. After the bacteria were incubated at 37°C for 48 h, and the fungi were incubated at 25°C for 72 h, the antimicrobial activity was determined according to the diameter of the inhibition zone around the disc.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). To measure the antimicrobial activity of a separated compound, the MIC (minimum inhibitory concentration) was determined through the broth dilution method (Sitohy et al., 2012). Each microbe was incubated in 1 ml broth with serially diluted separated compounds for 48−72 h and checked to see which concentration inhibited microbial growth. To confirm the MICs and to establish MBCs, 10 μl of broth was removed from each well and inoculated on tryptic soy agar and Sabouraud dextrose agar plates. After aerobic incubation at 37°C overnight, the number of surviving organisms was determined. The MIC was the lowest concentration which resulted in a significant decrease in inoculum viability, while the MBC was the concentration at which 99.9% or more of the initial inoculum was killed.

Manufacturing mesoporous ZnO particles. We synthesized mesoporous ZnO using the precipitation method in polyol solvent. To control the particle size easily, sodium hydroxide was added. The molar ratio of zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$, Mw=219.5, Samjun, Korea), precursor of zinc oxide and sodium hydroxide was 1:1. Diethylene glycol ($C_2H_{10}O_3$, Mw=106.12, Samjun) was used as a solvent. The reaction mixture was mixed under reflux conditions at 443 K. After the reaction was completed, the precipitates were recovered by washing with ethanol and centrifugation at 10,000 rpm.

Manufacturing mesoporous silicas. We synthesized mesoporous MCM-41 using a hydrothermal method (Park et

al., 2011). Two separate reactant formulations were used. The molar composition ratio of MCM-41-9 is 0.5-CTABr|1.0-SiO_2|0.3-Na_2O|70-H_2O and that of MCM-41-11 is 0.2-CTABr|1.0-SiO_2|0.3-Na_2O|70-H_2O. The reaction mixture was mixed at 373 K for 2 days. The solid product was recovered by filtration and dried overnight in an oven at 313 K. After drying, the samples were calcined at 823 K for 6 h (heating rate = 4.5 K/min) to remove the organic template by oxidation.

We also synthesized mesoporous SBA-15 using a Pluronic P123 as the structure-directing agent (Park et al., 2011). The molar composition ratio of SBA-15-2 is 0.017-P123|1.0-TEOS|6.0-HCl|196.0-H_2O and that of SBA-15-3 is 0.017-P123|0.74-TEOS|6.0HCl|196.0-H_2O. The resulting gel was aged at 373 K for 2 days. After aging, the solid was recovered, washed, and air-dried at room temperature. Calcination was carried out by increasing the temperature from room temperature to 823 K (4.5 K/min), and allowing cooling to 773 K followed by a 6-h soak at 773 K.

Incorporating RBI within the channel of mesoporous ZnO. To incorporate RBI in the mesoporous ZnO particles, RBI with 50% (w/v) concentration was mixed and rubbed for penetrating solution into the pore of mesoporous ZnO (Carmona et al., 2012). The amount of RBI adsorbed by each mesoporous ZnO was determined by observing sample stickiness. The RBI content of mesoporous ZnO measured by UV-vis spectrophotometry (CARY 300 Bio, Varian, Palo Alto, CA) and the amount RBI was 10% (w/v) (data not shown). The number of incorporations of organic substances within the channel of mesoporous ZnO was three cycles.

Characterization. We used Mass Spectrometry (G7001B, Agilent, Santa Clara, CA) to analyze two compounds isolated from *M. oleifera* seed extract. To confirm the condition of ZnO in the pores, X-ray diffraction (XRD, Cu-Kα X-ray, Rigaku D/MAX-III instrument, Tokyo, Japan) was used to determine the existence of crystals. The peak results of the XRD data of the mesoporous particles containing ZnO ions were compared. The morphology of the particulate ZnO was evaluated using a field emission scanning electron microscope (SEM, JSM-7401F, JEOL, Tokyo, Japan) or trans emission microscope (TEM, 300Kv, JEM2100F, JEOL). We measured the surface area and pore size of particles with a BET (Brunaure-Emmett-Teller) instrument (TriStar, Micromeritics, Norcross, GA) and the BJH (Barrett-Joyner-Halenda) method.

Results

Two compounds were isolated from EA fraction extracts of *M. oleifera* seed by preparative reverse-phase HPLC. UV, MS, ^{1}H, and ^{13}C-NMR spectral data of the two separated compounds correspond with the reported data (Cheenpracha et al., 2010; Eilert et al., 1981). Compounds 1 and 2 were thus identified as niazimicin and RBI from these data (Fig. 1).

The antimicrobial activity of the isolated niazimicin and RBI using the paper disc method and the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) against 3 bacteria and 2 fungi is shown in Table 1. In the results of the paper disc diffusion method and MIC test, RBI showed antimicrobial activity against all of the tested microorganisms and better activity compared with niazimicin. The MBC of RBI on *S. aureus*, *E. coli*, *P. aeruginosa, C. albicans* and *A. niger* was 0.1%, 0.5%, 1%, 1%, and 1%, respectively.

To investigate the synergistic antimicrobial efficacy through hybridized complex of organic-inorganic composite materials, RBI was loaded in the mesoporous silica (MCM-41/SBA-15) and zinc. We evaluated the antimicrobial effect of the mesoporous MCM-41/SBA-15/ZnO before and after RBI loading. Before loading RBI, MCM-41 and SBA-15 did not show activity against any of the tested

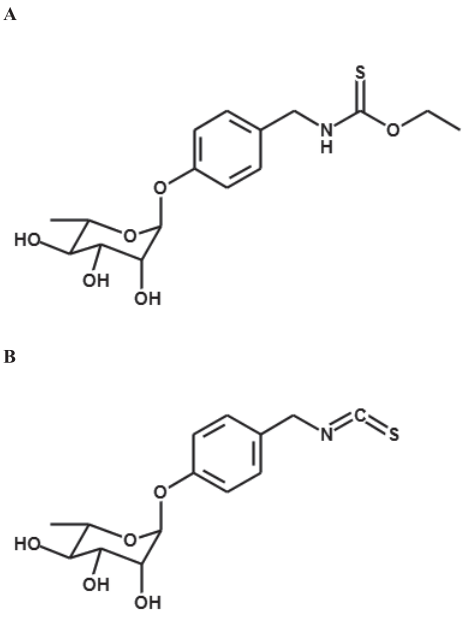

Fig. 1. Chemical structures of niazimicin (A) and RBI (B).

Table 1. In vitro antimicrobial activity of compounds separated from *Moringa oleifera* seed against a zone of growth inhibition and MIC and MBC of compounds separated from *Moringa oleifera* seed against bacteria and fungi.

Microorganism	Niazimicin		RBI		
	Inhibition zone (mm)_5 mg/disc	MIC (%)	Inhibition zone (mm)_5 mg/disc	MIC (%)	MBC (%)
Bacteria					
Staphylococcus aureus	23	0.1	28	0.005	0.1
Escherichia coli	10	—[a]	13	0.1	0.5
Pseudomonas aeruginosa	10	—[a]	11	0.5	1
Fungi					
Candida albicans	10	—[a]	16	0.5	1
Aspergillus niger	—[a]	—[a]	11	0.5	1

[a] No antimicrobial activity.

microorganisms (data not shown), but mesoporous ZnO showed good antimicrobial efficacy (Table 2). It was known that ZnO nanoparticles have antimicrobial activity (Jones et al., 2008; Raghupathi et al., 2011; Tong et al., 2013; Zhang et al., 2007, 2010). Nanoparticles that bind to cell surfaces can penetrate inside the bacteria by disturbing membrane permeability and kill by interacting with DNA and protein

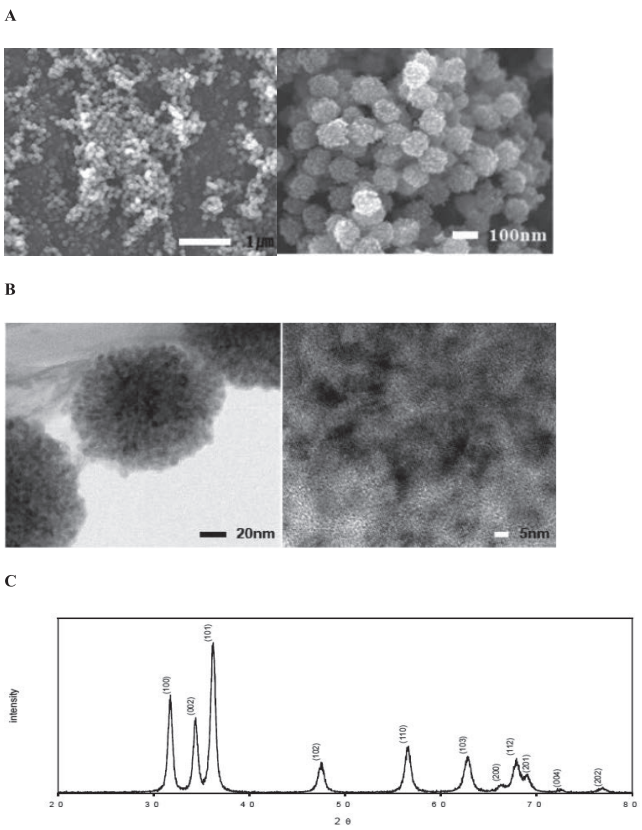

Fig. 2. SEM image (A) and TEM image (B) of porous zinc oxide, (C) XRD pattern of porous zinc oxide by the sol-gel method.

(Morones et al., 2005). Therefore, mesoporous ZnO was chosen as a carrier of RBI for enhanced antimicrobial efficacy.

The SEM image of zinc oxide particles revealed the shape of synthesized particles as spherical and particle size as about 100 nm uniformly (Fig. 2A). In the TEM image (Fig. 2B) the spherical zinc oxide particles were aggregates composed of very small nano particles of 10 nm. We also reconfirmed the particle size to be 100 nm in the TEM image, which was previously proven in the SEM image.

The XRD pattern of zinc oxide particles was compared and interpreted with standard data of the Joint Committee on Powder Diffraction Standards (JCPDS, 36-1451, a=3.429Å, c=5.206Å, λ=Cu 1.54Å). The ten characteristic peaks for zinc oxide particles appeared, which correspond to crystal facets of (100), (002), (101), (102), (110), (103), (200), (112), (201), (004) and (202) of zinc oxide (Fig. 2C). The XRD data and interpretation of zinc oxide revealed the structure of the particles prepared as hexagonal wurtzite.

The synergistic effect of RBI with mesoporous ZnO was investigated under the same conditions. As a result of MIC test, the complexed ZnO showed synergistic effects against all microbes tested (Table 3) compared with ZnO alone. These effects were prominent on *C. albicans* and *A. niger* because MIC was enhanced to 0.1% compared with ZnO alone, which had no inhibitory effects (Tables 2 and 3). Although the RBI concentration within mesoporous ZnO was one tenth that of RBI alone because the collection efficiency of RBI was 10% (w/v), the antimicrobial effects were significantly increased against all microbes tested with the incorporation in mesoporous ZnO.

Discussion

Nikkon et al. reported that the aglycone of deoxy-niazimicine isolated from the chloroform fraction of an ethanol

Table 2. Antimicrobial efficacy of porous zinc oxide at various concentrations.

Microorganism	Sample concentration % (w/v)					
	0.001	0.005	0.01	0.05	0.1	0.5
Bacteria						
Staphylococcus aureus	+	−	−	−	−	−
Escherichia coli	+	+	+	−	−	−
Pseudomonas aeruginosa	+	+	+	+	+	+
Fungi						
Candida albicans	+	+	+	+	+	+
Aspergillus niger	+	+	+	+	+	+

+ : growth ; − : inhibition.

Table 3. Antimicrobial efficacy of porous zinc oxide encapsulated with RBI at various concentrations.

Microorganism	Sample concentration % (w/v)					
	0.001	0.005	0.01	0.05	0.1	0.5
Bacteria						
Staphylococcus aureus	−	−	−	−	−	−
Escherichia coli	+	+	−	−	−	−
Pseudomonas aeruginosa	+	+	+	+	+	−
Fungi						
Candida albicans	+	+	+	+	−	−
Aspergillus niger	+	+	+	+	−	−

+ : growth ; − : inhibition.

extract of *Moringa oleifera* root bark was found to be responsible for the antibacterial and antifungal effects (Nikkon et al., 2003). We found that niazimicin also has anti-microbial activity on bacteria and fungi. Eilert et al. reported that RBI isolated from *Moringa oleifera* root extract has antimicrobial activity against *Bacillus subtilis*, *Mycobacterium phlei, Serratia marcescens, S. aureus*, *E. coli*, *P. aeruginosa, C. albicans,* and *A. niger* (Eilert et al., 1981).

From our results, RBI showed much better antimicrobial activity than niazimicin. The difference between the antimicrobial activity of RBI and niazimicin may be dependent on an ethyl group. RBI could more easily penetrate the cell membrane surface of microbes. We selected RBI as an incorporating agent on mesoporous ZnO based on antimicrobial activity.

We found that antibacterial activity increased in all tested microorganism by combining RBI and mesoporous ZnO. In a comparison of antimicrobial activity, the MIC of RBI with mesoporous ZnO was 5-fold lower than that of ZnO alone in *S. aureus* and *E. coli* and 50-fold lower in *S. aureus* and 100-fold lower in *E. coli* than that of RIB alone. While RBI with mesoporous ZnO showed antimicrobial activity in *P. aeruginosa* and two fungi, there was no antimicrobial activity for ZnO alone or 0.01% RBI which was encapsulated in mesoporous ZnO. Considering RBI content in mesoporous ZnO, RBI with mesoporous ZnO showed 10 to 100 times more antibacterial activity than RBI and ZnO alone.

Mesoporous ZnO not only acted as a carrier of RBI, but also acted as an antibacterial agent. It is known that ZnO nanoparticles produce reactive oxygen species (ROS) and damage cell membranes because of electrostatic interaction between ZnO and the cell membrane envelope structure (Raghupathi et al., 2011; Zhang et al., 2007, 2010). The synergistic effects of RBI and mesoporous ZnO suggest that the stability of RBI could be increased by incorporating RBI within mesoporous ZnO. The antimicrobial efficacy was also elevated by sustained slow release of RBI from mesoporous ZnO, which acts as a reservoir.

In conclusion, mesoporous ZnO loaded with RBI isolated from the *Moringa oleifera* seed has synergistic antimicrobial efficacy. From this result, mesoporous ZnO could s as a reservoir for RBI and complex ZnO with RBI could be used for cosmetic preservatives. Further studies are needed on the compatibility of this complex for cosmetic use.

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