Antimicrobial Isothiocyanates from the Seeds of *Moringa oleifera* Lam.

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Z. Naturforsch. **67c**, 557–564 (2012); received November 9, 2011/August 23, 2012

4-(α-L-Rhamnosyloxy)benzyl isothiocyanate (**1**) and 4-(4'-*O*-acetyl-α-L-rhamnosyloxy)-benzyl isothiocyanate (**2**) isolated from *Moringa oleifera* seeds were screened for their antibacterial activities against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, and for their antifungal activities against *Candida albicans*, *Trichophyton rubrum*, and *Epidermophyton floccosum* using the disk diffusion method. Isothiocyanates **1** and **2** were found active at the lowest inhibitory concentration of 1 mg/ml against all Gram-positive bacteria tested (*S. aureus*, *S. epidermidis*, *B. subtilis*) and against the dermatophytic fungi *E. floccosum* and *T. rubrum.* Statistically significant differences were found between the mean inhibition zones (IZ) of **1** and **2** and the standard drugs, ofloxacin and clotrimazole. The minimum inhibitory concentration (MIC) values confirmed the good antimicrobial activity of **1** and **2** against *S. aureus*, good to moderate activity against *S. epidermidis*, moderate activity against *B. subtilis*, and weak activity against *E. floccosum* and *T. rubrum*. The *in vitro* bactericidal effect of **1** and **2** against the Gram-positive bacterial strains tested is suggested by MBC:MIC ratios of 2:1.

Key words: Moringa oleifera, Isothiocyanates, Antimicrobial

Introduction

Moringa oleifera has long been valued as both a food and medicinal tree. The therapeutic benefits, for which different tree parts are used, include analgesic (Sutar *et al*., 2008), antipyretic (Oliveira *et al*., 1999), hypocholesterolemic (Mehta *et al*., 2003; Ghasi *et al*., 2000), hypoglycemic (Makonnen *et al*., 1997), hepatoprotective (Pari and Kumar, 2002), antihypertensive (Faizi *et al*., 1995), anti-inflammatory (Ezeamuzle *et al*., 1996), antispasmodic (Caceres *et al*., 1992), anti-ulcer (Pal *et al*., 1995), antioxidant (Siddhuraju and Becker, 2003), anticonvulsant (Amrutia *et al*., 2011), antimicrobial (Caceres *et al*., 1991), and antitumour (Guevara *et al*., 1999; Murakami *et al*., 1998) activities. The seeds of *M. oleifera* yielded 4-(α-L-rhamnosyloxy)benzyl isothiocyanate which exhibited a minimum bactericidal concentration of 40 μM for *Mycobacterium phlei* and 56 μM for *Bacillus subtilis* (Eilert *et al*., 1981). A new nitrile has also been reported from *M. oleifera* (Faizi *et al*., 1994) which is of relevance to our present report. We earlier reported the cytotoxicity of 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate (**1**) and 4-(4'-*O*-acetyl-α-L-rhamnosyloxy)benzyl isothiocyanate (**2**) (Fig. 1) isolated from the seeds of *M. oleifera* (Ragasa *et al*., 2012). We report herein the antimicrobial properties of **1** and **2**.

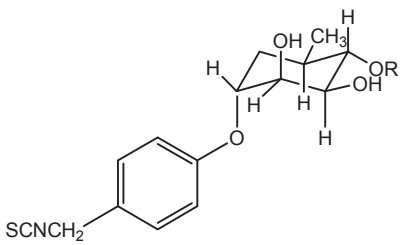

Fig. 1. Chemical structures of **1** (R = H) and **2** (R = Ac).

Material and Methods

General experimental procedures

NMR spectra were recorded on a Varian (Palo Alto, CA, USA) VNMRS spectrometer in $CDCl_3$, ^1H NMR spectra at 600 MHz and ^{13}C NMR spectra at 150 MHz. Two-dimensional (2D) NMR data (COSY, HSQC, and HMBC) were obtained for **1** and **2**. EI- and ESI-mass spectra were recorded on Finnigan (San Jose, CA, USA) GCQ and LCQ spectrometers, respectively. Column chromatography (CC) was performed with silica gel 60 (70–230 mesh). Thin-layer chromatography (TLC) was performed with plastic backed plates coated with silica gel F_{254} (Merck, Darmstadt, Germany). Plates were visualized by spraying with vanillin/sulfuric acid and warming.

Plant material

Fresh leaves and fruits of *Moringa oleifera* Lam. were collected from Ibaan and Tanauan City, Batangas, Philippines, respectively, in May 2010. The samples were authenticated at the Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines, Diliman, Quezon City, Philippines. A voucher specimen (#198) was deposited at the Chemistry Department, De La Salle University, Manila, Philippines.

Extraction and isolation

Fresh seeds of *M. oleifera* were ground in an Osterizer blender (Ciudad de Mexico, Mexico) and then freeze-dried. The freeze-dried seeds (103.5 g) were soaked in CH_2Cl_2 for 3 d and then filtered. The filtrate was concentrated *in vacuo* to afford a crude extract (7.9 g). A 51 x 5.1 cm glass column was packed with silica gel at a ratio of crude extract to silica gel of 1:25 (w/w). The crude extract was fractionated by elution with increasing proportions of acetone in CH_2Cl_2 (10% increment by volume). One hundred-ml fractions were collected. All fractions were monitored by TLC. Fractions with spots of the same R_f values were combined and rechromatographed in appropriate solvent systems until TLC-pure isolates were obtained. A 37 x 1.8 cm glass column was used to rechromatograph the 60%–80% acetone/CH_2Cl_2 fractions using CH_2Cl_2/Et_2O/CH_3CN (2:2:6, v/v/v) as eluent. Ten-ml fractions were collected. Final purification was achieved using a 15 x 0.5 cm glass column. One-ml fractions were collected. The less polar fractions were rechromatographed (3x) using CH_2Cl_2/Et_2O/CH_3CN (2:2:6, v/v/v) as eluent to afford **2** [95 mg, $[\alpha_D] = -126.5°$ (*c* 0.9, $CHCl_3$)] after washing with Et_2O. The more polar fractions were rechromatographed (3x) with CH_2Cl_2/Et_2O/CH_3CN (2.5:2.5:5, v/v/v) to afford **1** [125 mg, $[\alpha_D] = -106.5°$ (*c* 1.3, $CHCl_3$)] after washing with Et_2O.

Microbial strains

The microbial strains used in the study were obtained from the Natural Sciences Research Institute, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines, College of Public Health (Manila, Philippines), and from the De La Salle Health Sciences Institute (Dasmariñas, Cavite, Philippines).

The inhibitory activities of compounds **1** and **2** were determined against 7 bacterial and 3 fungal strains, respectively. The bacteria were: *Staphylococcus aureus* (ATTC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 8739), *Enterobacter aerogenes* (ATCC 13048), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 9027), and *Bacillus subtilis* (ATCC 6633). The fungal strains were *Candida albicans* (ATCC 10231) and two dermatophytic fungi, *Trichophyton rubrum* and *Epidermophyton floccosum.*

Bacterial and fungal stock cultures were maintained in nutrient agar (Difco, Sparks, MD, USA) and Sabouraud dextrose agar (SDA; Difco) slants, respectively, and kept refrigerated until use. All microbial cultures were checked for purity by plating out prior to testing. Except for the dermatophytic fungi [which were incubated at (26 ± 2) °C], the cultures were incubated at (35 ± 2) °C.

Antibiotic disks and standard antimicrobials

Standard antibacterial and antifungal agents, ofloxacin (Sigma-Aldrich, St. Louis, MO, USA) and clotrimazole (Sigma-Aldrich), were utilized in the disk diffusion susceptibility testing of the bacterial and fungal test strains, respectively, and in the determination of the MIC values of the test compounds. Prior to dilution with sterile distilled water to the desired concentration, ofloxacin and clotrimazole required solubilization with 0.1 M sodium hydroxide (NaOH) and dimethyl sulfoxide (DMSO), respectively.

Preparation of inoculum

For each bacterial test strain, 4–5 colonies from a nutrient agar plate were transferred to 5 ml nutrient broth (Difco). After 18–24 h incubation, the inoculum density was adjusted with sterile normal saline solution (NSS) to match the McFarland 0.5 turbidity standard (10^8 CFU/ml). To prepare the *C. albicans* inoculum, 4–5 colonies from a SDA plate were picked and suspended in 5 ml sterile NSS. The suspension was vortex-mixed and the cell density was adjusted to that of McFarland 0.5 standard. The dermatophyte inoculum was prepared by adding sterile NSS to a 5- to 10-day-old SDA slant culture. The conidia were dislodged from the hyphal mat using a sterile inoculating loop, after which the suspended cells were carefully pipetted into sterile tubes. The cell density was subsequently adjusted with sterile NSS to a final inoculum concentration equivalent to McFarland 0.5 standard.

Preparation and seeding of double layer Mueller Hinton agar (MHA) plates

Double layer MHA (Difco) plates consisting of 10 ml MHA base and 5 ml upper seeded layer were used in this study. The upper seed layer was made by inoculating a tube containing 5 ml of sterile, molten MHA with 0.1 ml of a standardized inoculum (10^8 CFU/ml). After a quick mix, the seeded molten medium was poured into an MHA base plate, and was allowed to solidify before diffusion disks were applied.

Antimicrobial assay

The evaluation of the antibacterial and antifungal activities of the isothiocyanates **1** and **2** was conducted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009). Using the disk diffusion method the test compounds were dissolved in 95% ethanol and diluted with sterile NSS to obtain 4 serial tenfold dilutions: 10 mg/ml, 1 mg/ml, 100 μg/ml, and 10 μg/ml.

To a plate seeded with one of the test organisms, four 6-mm antibiotic disks (impregnated with 20 μl of each of the dilutions) and a solvent blank (impregnated with 20 μl of the solvent) were applied. A standard antibiotic disk and a disk impregnated with 20 μl of sterile distilled water were likewise applied as positive and negative controls, respectively. After incubation (18–24 h for bacteria, 48–72 h for *C. albicans*, and 5–10 d for the dermatophytic fungi), the antimicrobial activity was assessed on the basis of the inhibition zone (IZ) size. The endpoint was taken as complete inhibition of growth as judged by the naked eye. The diameters of the zones of inhibition were measured in millimeters (including the diameter of the disk) using a Vernier caliper. Values were the average of three readings. Inhibition zones equal to or greater than 7 mm were considered indicative of the antimicrobial activity of the test compound against the respective test organism. Negative results were recorded as zero.

The activity indices (AIs) of the test compounds were obtained by dividing their zone of inhibition by that of the standard antimicrobial agent. An AI > 0.5 was considered as significant antimicrobial activity.

Determination of minimum inhibitory concentration (MIC)

The MIC values of the test compounds were determined by the broth dilution method for all test strains which produced inhibition zones > 7 mm. Mueller Hinton broth (Difco) and Sabouraud dextrose broth (Difco) were used to prepare the dilutions for the bacterial and fungal test strains, respectively.

Five two-fold serial dilutions of the test compounds were set up with respective upper and lower concentrations of 2 mg/ml and 125 μg/ml for bacteria and 20 mg/ml and 1.25 mg/ml for fungi. The total volume of the solutions prepared was adjusted to the number of organisms to be tested. Standard antimicrobials (ofloxacin and clotrimazole) were set up in a similar manner.

To each of the dilution and control tubes containing broth only, an equal volume of standardized inoculum (10^8 CFU/ml) was added to give respective final upper and lower concentrations of 1 mg/ml and 62.5 μg/ml for bacteria and 10 mg/ml and 625 μg/ml for fungi. At the end of the incubation period (18–24 h for bacteria, 5–10 d for dermatophytic fungi), the tube containing the lowest concentration of the test compound showing no visible sign of growth was considered as the MIC against the test organism. All MIC determinations were the average of two readings.

MIC values < 100 μg/ml were considered having good antimicrobial activity; MIC values of

100–500 μg/ml having moderate activity; MIC values of 0.5–1 mg/ml having weak activity; and MIC values > 1 mg/ml having no activity.

Determination of minimum bactericidal concentration (MBC)/minimum fungicidal concentration (MFC)

For MBC/MFC determinations, 0.1 ml from MIC tubes which did not show any sign of growth was inoculated onto MHA (for bacteria) and SDA (for fungi) by the spread plate method. After incubation (18–24 h for bacteria, 5–10 d for dermatophytic fungi), the lowest concentration of the test compound with no visible growth on subculture was taken as its MBC/MFC against the respective test organism.

MBC:MIC or MFC:MIC ratios were calculated to determine the antibacterial or antifungal effect of the test compounds against the test organisms. If the ratio is between 1:2 to 2:1, the compound is considered bactericidal or fungicidal against the test organism, or is likely to be bacteriostatic or fungistatic if the ratio is higher than 2:1.

Statistical analysis

Means and standard deviations (SD) of the IZs of the test compounds and standard antimicrobials against the test strains were calculated. ANOVA was used to determine whether there are statistically significant differences among the inhibitory concentrations (10 mg/ml and 1 mg/ml) of the test compounds and the standard antimicrobials. ANOVA p-values less than 0.05 were considered statistically significant. Furthermore, when the p-value of the F-test was smaller than the 0.05 level of significance, Dunnet's test was used to determine which inhibitory concentration of the test compound is significantly different from the standard antimicrobial. The means and standard deviations of the activity indices of the test compounds against the test strains were calculated as well. Post-hoc comparison tests were performed using Scheffe's method.

Results and Discussion

*Isolation and identification of **1** and **2***

The dichloromethane extract of the freeze-dried seeds of *M. oleifera* afforded the isothiocyanates **1** and **2** by silica gel chromatography. Their structures were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{1}H NMR and ^{13}C NMR data with those reported in the literature for 4-(α-L-rhamnosyloxy)benzyl isothiocyanate (**1**) (Eilert *et al*., 1981) and 4-(4α-*O*-acetyl-α-L-rhamnosyloxy)-benzyl isothiocyanate (**2**) (Faizi *et al*., 1994).

Antimicrobial assay

Table I summarizes the inhibitory activities of the test compounds **1** and **2** against 7 bacterial and 3 fungal test strains as determined by the disk diffusion method. **1** and **2** exhibited antibacterial activity against the three Gram-positive bacteria at concentrations ≥1 mg/ml, but were inactive against the four Gram-negative bacteria even at the highest concentration used (10 mg/ml). Amongst the Gram-positive bacteria, *S. aureus* was found to be the most susceptible and *B. subtilis* the least susceptible. Of the three fungal species, only *C. albicans* failed to exhibit susceptibility to **1** and **2** even at 10 mg/ml. Of the dermatophytic fungi, *T. rubrum* was more susceptible to **1**, while *E. floccosum* was more susceptible to **2**. The data also show that the mean inhibition zones (IZs) of **1** and **2** were smaller than those of the standard drugs, and ANOVA (all $p < 0.05$) indicated statistically significant differences in the mean IZs against all susceptible test strains.

Table I likewise shows the activity indices (AIs) of the different concentrations of the test compounds. The results indicate that relative to the activity of ofloxacin, **1** and **2** exhibited the greatest antibacterial activity against *S. aureus* and the least activity against *B. subtilis*. The results further illustrate that the AIs of **1** and **2** at 10 mg/ml and 1 mg/ml were highest against *S. aureus* and lowest against *E. floccosum*. Based on an AI cut-off value of >0.5, significant inhibitory activities were exhibited by **1** and **2** against *S. aureus* at 1 mg/ml; and against *S. aureus*, *S. epidermidis*, *B. subtilis*, and *T. rubrum* at 10 mg/ml. No significant inhibitory activity was exhibited by both compounds against *E. floccosum* at 1 and 10 mg/ml.

The negative control (sterile distilled water) and solvent blanks (0.1 M NaOH and DMSO) showed no inhibitory activity against any of the microorganisms tested.

The results of post-hoc comparison tests of the mean IZs between the test compounds and standard drugs reveal that the least computed mean difference was against *S. aureus* between **1** at

Table I. Disk diffusion-based inhibitory activities of compounds **1** and **2** against 10 microbial strains.

Bacterium or fungus	Compound	10 mg/ml		1 mg/ml		100 μg/ml		10 μg/ml		Ofloxacin (30 μg/ml)	Clotrimazole (30 μg/ml)
		IZ (SD)	AI (SD)	IZ (SD)	AI (SD)	IZ	AI	IZ	AI	IZ (SD)	IZ (SD)
S. aureus	**1**	21.13 (1.48)	0.75 (0.06)	16.03 (1.27)	0.57 (0.03)	0	0	0	0	28.13 (0.76)	-
	2	23.10 (0.44)	0.82 (0.01)	18.10 (0.53)	0.64 (0.04)	0	0	0	0	28.13 (0.76)	-
S. epidermidis	**1**	19.70 (0.95)	0.58 (0.02)	15.50 (2.00)	0.45 (0.06)	0	0	0	0	34.10 (1.65)	-
	2	22.00 (0.50)	0.65 (0.03)	17.07 (0.75)	0.50 (0.04)	0	0	0	0	34.10 (1.65)	-
B. subtilis	**1**	14.23 (0.84)	0.57 (0.04)	8.50 (0.75)	0.34 (0.04)	0	0	0	0	25.17 (0.76)	-
	2	14.50 (1.04)	0.58 (0.06)	9.10 (0.79)	0.36 (0.02)	0	0	0	0	25.17 (0.76)	-
E. coli	**1**	0	0	0	0	0	0	0	0	40.4 (0.53)	-
	2	0	0	0	0	0	0	0	0	40.4 (0.53)	-
E. aerogenes	**1**	0	0	0	0	0	0	0	0	21.63 (0.23)	-
	2	0	0	0	0	0	0	0	0	21.63 (0.23)	-
K. pneumoniae	**1**	0	0	0	0	0	0	0	0	38.33 (1.15)	-
	2	0	0	0	0	0	0	0	0	38.33 (1.15)	-
P. aeruginosa	**1**	0	0	0	0	0	0	0	0	28.00 (1.00)	-
	2	0	0	0	0	0	0	0	0	28.00 (1.00)	-
C. albicans	**1**	0	0	0	0	0	0	0	0	-	17.07 (1.27)
	2	0	0	0	0	0	0	0	0	-	17.07 (1.27)
E. floccosum	**1**	10.83 (2.25)	0.32 (0.07)	7.03 (0.47)	0.20 (0.01)	0	0	0	0	-	34.53 (1.29)
	2	13.00 (1.83)	0.38 (0.06)	8.47 (0.50)	0.25 (0.02)	0	0	0	0	-	34.53 (1.29)
T. rubrum	**1**	11.00 (0.92)	0.54 (0.03)	7.20 (1.11)	0.35 (0.05)	0	0	0	0	-	20.47 (0.50)
	2	12.23 (0.80)	0.60 (0.03)	7.53 (0.42)	0.37 (0.02)	0	0	0	0	-	20.47 (0.50)

IZ (inhibition zone), in millimeters, mean of triplicate readings ± SD (standard deviation); AI (activity index) = IZ of compound/IZ of standard drug.

10 mg/ml and ofloxacin, while the greatest computed mean difference was against *E. floccosum* between **1** at 1 mg/ml and clotrimazole. Similar results were observed for compound **2**. All of the Dunnet's test's p-values were smaller than the 0.05 level of significance, thus all mean differences in IZ between test compounds and standard drugs were statistically significant.

The results of post-hoc comparison tests of the mean differences in IZs of **1** and **2** indicate that for all 5 test strains, the mean differences in IZs between **1** and **2** at 1 mg/ml were not statistically significant. Similarly, no significant difference was observed in the mean differences in IZs between **1** and **2** at 10 mg/ml. Except for *E. floccosum*, the mean differences in IZs of **1** at 1 mg/ml were significantly different from those obtained at 10 mg/ml; but for all test strains, the mean differences in IZs of **2** at 1 mg/ml were significantly different from those obtained at 10 mg/ml. For *S. aureus*, *B. subtilis*, and *T. rubrum*, the mean differences in IZs of **1** and **2** at 1 mg/ml were significantly different from those obtained at 10 mg/ml. For *S. epidermidis* and *E. floccosum*, the mean differences in IZs of **1** at 1 mg/ml were significantly different from those of **2** at 10 mg/ml; however, the mean differences in IZs of **2** at 1 mg/ml were not significantly different from those of **1** at 10 mg/ml.

Table II shows the parameters of antimicrobial activity (MIC and MBC) of **1** and **2**. The results of the broth dilution MIC determination indicate that **1** exhibited good antibacterial activity against *S. aureus* and moderate activity against *S. epidermidis* and *B. subtilis*. While **2** was also moderately active against *B. subtilis*, it showed good antibacterial activity against *S. aureus* and *S. epidermidis*. Both **1** and **2** exhibited weak activity (MIC 1 mg/ml) against the dermatophytic fungi *E. floccosum* and *T. rubrum*.

The MBC values of **1** and **2** were lowest for staphylococci, specifically for *S. aureus*. Both compounds had the same MFC values against the dermatophytic fungi tested. The apparent higher MBC values (by one dilution step) compared to the corresponding MIC values suggest that the bactericidal activities of **1** and **2** occur at concentrations higher than their growth inhibitory concentrations.

As indicated by MBC:MIC ratios of 2:1, **1** clearly exerted bactericidal activity against *S. aureus*, *S. epidermidis*, and *B. subtilis*. Although the bactericidal effect of **2** was assessable only against *S. epidermidis* and *B. subtilis*, a parallel lethal action against *S. aureus* is clearly possible considering that **1** and **2**, based on their MIC values, shared the same antimicrobial activity against the organism. The calculation of MFC:MIC ratios of **1** and **2** against *E. floccosum* and *T. rubrum* was not possible since the compounds' MFC values against these fungi exceeded the highest concentration tested (1 mg/ml).

This study has shown that **1** and **2** possess specific antibacterial activity towards the Gram-positive organisms tested (*S. aureus*, *S. epidermidis*, and *Bacillus subtilis*). This finding is congruent with results from other studies which demonstrated that plant-derived compounds are more effective against Gram-positive organisms than Gram-negative ones (Cos *et al*., 2002; Rabe and van Staden, 1997; Taylor *et al*., 1995). Differences in cell wall structures and cell membrane permeability have been suggested as possible explanations for this susceptibility pattern (Duffy and Power, 2001; Schaechter *et al*., 1999). More importantly, the finding that **1** and **2** possess significant antistaphylococcal property validates the plant's long established use and current applications.

The antifungal activities of **1** and **2** did not match those of the compounds' antibacterial potential. The greater resistance of the fungal strains

Table II. Parameters of antimicrobial activity of compounds **1** and **2**.

Test bacterium	**1**			**2**		
	MIC [μg/ml]	MBC [μg/ml]	MBC: MIC ratio	MIC [μg/ml]	MBC [μg/ml]	MBC: MIC ratio
S. aureus	62.5	125	2:1	<62.5	<62.5	NC
S. epidermidis	125	250	2:1	62.5	125	2:1
B. subtilis	250	500	2:1	250	500	2:1
Test fungus	MIC [mg/ml]	MFC [mg/ml]	MFC: MIC ratio	MIC [mg/ml]	MFC [mg/ml]	MFC: MIC ratio
E. floccosum	1	(>1)	NC	1	(>1)	NC
T. rubrum	1	(>1)	NC	1	(>1)	NC

MIC and MBC (or MFC) values represent the average of two readings.
NC, not calculable.

to **1** and **2** may not only be explained by the differences in cellular makeup between fungi and bacteria, but also by the difference in the compounds' target structure and inhibitory mechanism (Ghannoum and Rice, 1999). Further studies are needed to support and expand upon these findings.

An earlier study (Eilert *et al*., 1981) reported the antibiotic activity of the aqueous extract from the seeds of *M. oleifera*. 4-(α-L-Rhamnosyloxy)-benzyl isothiocyanate isolated from defatted seeds exhibited antibacterial activity against *Bacillus subtilis* and *Mycobacterium phlei* with MBC values of 17.5 and 12.5 μg/ml, respectively, while it was inactive against *Serratia marcescens* (Eilert *et al*., 1981).

Acknowledgement

The authors gratefully acknowledge the financial support from the Health Research Development Consortium-Region IV for the antimicrobial assays, and the research grant from De La Salle Lipa for the isolation of the isothiocyanates. The kind assistance of the Center for Basic Biomedical Research-De La Salle Health Science Institute and the College of Science-De La Salle University is likewise appreciatively acknowledged.

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