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Full Length Research Paper

Novel *in-vitro* antimicrobial activity of *Vigna radiata* (L.) R. Wilczek against highly resistant bacterial and fungal pathogens

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The ever rising resistant bacteria and fungi resulted in finding novel antimicrobial sources and agents. Studies confirmed that mung beans have increased phenolic compounds and enhanced defenses during germination. We hypothesized that antimicrobial activities might be found in sprouts of mung beans (MBS), or *Vigna radiata* (L.) R. Wilczek. The screening method was conducted using disc diffusion assay against 12 gram negative and positive bacteria, including multiple drug resistant (MDR) bacteria and 12 fungi. It was followed by the evaluation of the minimum inhibitory concentration and the minimum bactericidal concentration or the minimum fungicidal concentration. The screening results revealed potential antibacterial and antifungal activities by MBS extract against 11 out of 12 bacteria and 2 out of 10 fungi including remarkable antimicrobial activity against highly infectious MDR bugs such as Methicilline-resistant *Staphylococcus aureus*, MDR *Escherichia coli* O157:H7, MDR *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *S. aureus*, and *Salmonella* Typhimurium as well as against human fungal pathogens, *Trichophyton rubrum* and *Trichoderma harzianum*. The potential antimicrobial activity of MBS reflects effective quality and quantity of polyphenolic compounds present after bean germination. This unprecedented study showed that MBS extract is a potential source for novel antimicrobials that are inexpensive and readily available at a large scale for pharmaceutical companies.

Key words: Antimicrobial, antifungal, multiple drug resistant, mung bean sprout, polyphenols.

INTRODUCTION

The continuous escalation of resistant bacteria and fungi against a wide range of antibiotics necessitates discovering novel unconventional sources of antibiotics. As a result of the indiscriminate use of antimicrobial drugs in the treatment of infectious diseases, microorganisms have developed resistance to many antibiotics (Cowan, 1999). In the recent world, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* O157:H7, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa* are considered prototype examples for the most notorious bugs of human population (Tateda and Ishii, 2003). Therefore, there is a critical need to move fast and develop alternative antimicrobial drugs. One approach is to screen local medicinal plants which represent a rich source of novel

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antimicrobial agents. Medicinal plants are an integral component of research development in microbiology and pharmaceutical industry. Natural products from plants traditionally have provided the pharmaceutical industry with one of its most important sources of lead compounds and up to 40% of modern drugs are derived from natural sources, using either the natural substance or a synthesized version (Gautam et al., 2007). Mung bean sprout (MBS), (*Vigna radiata* (L.) R. Wilczek), or previously known as *Phaseolus radiatus*, which is popular in Asian cuisine, is an excellent source of vitamins, minerals and protein with its essential amino acid profile comparable to that of soybean and kidney bean (Mubarak, 2005). The mung bean contains significant quantities of phenolic and polyphenolic compounds such as phenolic acids and flavonoids (Dabrowski and Sosulski, 1984a, b).

In the natural environment, seed sprouts survive during germination by enhancing their defensive responses through phenolics biosynthesis including modified vitamins, enzymes, and receptors etc. (Randhir et al., 2004). Among the enhanced defensive mechanisms during germination, the antimicrobial defenses might be highly involved. However, the antimicrobial defenses were not covered adequately in germinated sprouts in most of medicinal plants. Plant phenolic metabolites are gaining interest due to their potential role in human disease prevention and treatment. The use of phytochemicals as natural antimicrobial agent commonly called 'biocides' are gaining popularity (Smid and Gorris, 1999). The main advantage of natural phenolic agents from plants is that they might contain a spectrum of phenolic antimicrobials, not only single antimicrobial substance, directed toward certain spectrum of microbes which potentially do not enhance the 'antibiotic resistance' phenomenon commonly seen with long-term use of synthetic antibiotics (Randhir et al., 2004). Hence, some plant phenolics are being developed as potential antimicrobial agents and used in the defense against human pathogens (Nychas et al., 2003; Tranter et al., 1993). Unfortunately, no previous studies scrutinized the possible enhancement of natural antimicrobial defenses during germination. Moreover, to the best of our knowledge, there was no previous comprehensive study conducted specifically to evaluate the antimicrobial activity of MBS against different notorious human bacterial and fungal pathogens. We hypothesized that there is a good possibility to find potential antimicrobial activity in MBS based on the enhanced antimicrobial defenses during germination.

Accordingly, this study was designed to test the antimicrobial and antifungal activity of MBS against 22 microbes. Twelve bacteria and twelve fungi including some notorious multiple drug resistant (MDR) bugs such as, for bacteria, Methicillin resistant *S. aureus* (MRSA), *E. coli* O157:H7, *P. aeruginosa*, and for fungi, *Trichoderma harzianum* and *Trichophyton rubrum*.

MATERIALS AND METHODS

Plant material and preparation of the extract

Fresh MBS, devoid of any preservative antimicrobials, was purchased from local markets in Selangor state in Malaysia. The sprouts were left to dry under room temperature at dark area for approximately 7 days. After sprouts dryness, they were grounded to powder. The grounded powder was extracted (1/10) with 1:1 v/v 2.4 mol/L HCl acidified aqueous methanol (Merck, Darmstadt, Germany) to extract all components of phenolic compounds, free and conjugated (Stratil et al., 2006) and soaked for three days in dark at room temperature. The solvent was then removed by filtration and fresh solvent was then added to the plant material. The extraction was repeated twice and extracts were combined and evaporated to dryness under vacuum at 40°C. The pH of MBS extract ranged from 6.8 to 7.0 which is a neutral pH. The powder was then stored at −18°C in a desiccant until required.

Microorganisms and medium

A total of 22 microorganisms of 12 bacteria and 10 fungi were used in this study (Tables 1 and 3). All microorganisms were obtained from the microbiology laboratory of the institute of Bioscience, University of Putra Malaysia. The bacteria other than ATCC strains were identified by the use of biochemical profiles according to the recommendations of the Manual of Clinical Microbiology (Murray and Baron, 2007) while the fungi other than ATCC strains were identified depending on the macro and microscopic features (Ulloa and Hanlin, 2000). The bacteria and fungi were maintained on nutrient agar slants (Merck, Darmstadt, Germany) and sabouraud dextrose agar slants (Merck, Darmstadt, Germany), respectively, at 4°C until required. Among the 12 bacteria tested in the current study, 3 MDR bacteria were selected out of a large number of isolates being subjected to a panel of antibiotic sensitivity tests. The MDR bacteria were MRSA, *P. aeruginosa*, and Enterohemorrhagic (EHEC) *E. coli* O157:H7 which were highly resistant to > 4 antibiotics. MRSA isolate was completely resistant to methicillin, erythromycin, streptomycin, clindamycin and tetracycline. Moreover, the MRSA isolate was a bit borderline resistant to vancomycin namely vancomycin-intermediate *S. aureus* (VISA). The MDR *P. aeruginosa* isolate was resistant to ceftazidime, ciprofloxacin, cefepime, imipenem, and piperacillin-tazobactam. The MDR *E. coli* O157:H7 isolate was resistant to ampicillin, kanamycin, sulfisoxazole, and tetracycline. The MDR *E. coli* O157:H7 was isolated from ground beef and it was confirmed to be EHEC via sorbitol MacConkey agar and verotoxin 1 and 2 immunoassays. Therefore, 9 non-MDR and 3 MDR bacteria were used to test the antibacterial effect of MBS.

Antimicrobial sensitivity tests

Disc diffusion assay

The dried plant extracts were dissolved in the same solvent, methanol, to a final concentration of 200 and 500 mg/ml for antibacterial tests and 200, 500 and 700 mg/ml for antifungal tests and were sterilized by filtration through 0.45 µm Millipore filters (Nalgene, UK). The primary screening antimicrobial test was carried out by disc diffusion (Murray and Baron, 2007) using 100 µl of suspension containing 10^8 CFU/ml of bacteria and 10^5 spore/ml of fungi, spread evenly on the surface of the nutrient agar and sabouraud dextrose agar plates, respectively. Sterile Whatman number 1 filter paper (MACHEREY-NAGEL, MN 615, Germany) was used to prepare 6 mm in diameter discs. These discs were processed, in triplicates, to contain 10 µl, that is; (2 mg/disc),

Table 1. Antibacterial activity of mung bean sprout methanol extract by disc diffusion method.

Microorganisms		Inhibition zone in diameter (mm)			
Bacterial species	**Number of strains**	***Vigna mungo* methanol extract**		**Negative control (MeOH*)**	**Positive control (Streptomycin10 µg/disc)**
		200 mg/ml	**500 mg/ml**		
Staphylococcus aureus	2 (clinical isolate)	16 ± 1.5	20 ± 1.2	- †	18 ± 0.4
S. aureus	1 (ATCC 29247 strain)	7 ± 0.9	17 ± 1.3	-	17 ± 0.8
MRSA [a]	1 (clinical isolate)	7 ± 0.8	17 ± 0.5	-	-
Bacillus subtilis	1 (clinical isolate)	10 ± 0.8	15 ± 1.0	-	18 ± 1.1
Escherichia coli	1(clinical isolate)	14 ± 0.8	16 ± 1.6	-	16 ± 0.9
E. coli	1 (ATCC 25922 strain)	15 ± 1.2	21 ± 1.5	-	11 ± 0.3
E. coli	1 (ATCC 35218 strain)	16 ± 0.8	19 ± 1.7	-	16 ± 0.5
E. coli O157:H7	1 (clinical isolate)	14 ± 1.1	17 ± 1.0	-	18 ± 0.8
Pseudomonas aeruginosa	1 (clinical isolate)	-	19 ± 1.2	-	18 ± 1.0
Klebsiella pneumoniae	1 (clinical isolate)	12 ± 1.0	20 ± 1.8	-	19 ± 0.7
Salmonella enterica serovar Typhimurium	*1 (ATCC 25241 strain)*	*13 ± 1.3*	*17 ± 2.3*	-	*15 ± 0.5*

*MeOH: methanol, †: (-) means no growth inhibition zone, [a] : Methicillin-resistant *Staphyloccocus aureus*.

(5 mg/disc) and (7mg/disc) to concentrations of (200 mg/ml), (500 mg/ml) and (700 mg/ml), respectively, and were then impregnated in the inoculated agar. Negative controls were prepared, in triplicates per Petri dish, using the same solvents employed to dissolve the plant extracts. Streptomycin "CALBIOCHEM, China" (10 µg/disc) and Amphotericin B "Sigma, Steinheim, Germany" (10 µg/disc) were used, in triplicates per Petri dish as positive control or reference standard drug to determine the sensitivity of each tested bacterium and fungus respectively towards the used extract in comparison with the positive control. For bacteria, the inoculated plates were incubated for 24 h at 37°C and, for fungi, were incubated for 3-5 days at 30-35°C. Clear inhibition zones around discs indicated the presence of antimicrobial activity. For optimal fidelity of results, the disc diffusion assay was repeated three times. Therefore, the mean ± SD was measured out of totally 9 inhibition zones, triplicate in each run.

Microdilution assay

The minimum inhibitory concentration (MIC) values were also studied for the microorganisms determined as sensitive to the extract by the disc diffusion assay. The microdilution method was used according to the methodology referred by Zgoda and Porter (2001) with some modifications. The inocula of the bacteria were prepared from 12 h broth cultures and standardized to 10^8 CFU/ml, while the fungal inocula were prepared from fresh fungal cultures 3-5 days according to the fungus type and standardized to 10^5 spore/ml. The stock extracts of 1200 and 700 mg/ml concentrations were prepared in 10% dimethylsulfoxide (Merck, Darmstadt, Germany). For bacteria, the extract solution was diluted using nutrient broth (Merck, Darmstadt, Germany) and, for fungi, the extract solution was diluted in sabouraud dextrose broth (Merck, Darmstadt, Germany).

Serial dilutions of the plant stock extract solutions were prepared in concentrations range from (0 to 600 mg/ml) for bacteria and (0 to 350 mg/ml) for fungi in 1.5 ml test tubes (Eppendrof, Hamberg, Germany). One hundred microliter per well of each microorganism suspension were dispensed, in triplicates, into 96-well microtiter plate (Steriline, UK). On the other hand, triplicates of extract-free bacterial and fungal spore suspensions were used as negative controls as well as triplicates of streptomycin at concentration range of (0.02-1 µg/ml) and Amphotericin B at concentration range of (0.5-3 µg/ml) were used as standard antibacterial and antifungal positive controls. The final volume in each well was 200 µl.

The plate was covered with a sterile plate sealer 80/140 mm (Greiner bio-one, Germany). For bacteria, plates were incubated for 24 h at 37°C and for fungi plates were incubated for 3 to 5 days at 30-35°C. The growth of bacteria and fungi was determined by absorbance values at 600 nm and 530 nm, respectively using fully automated Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, Canada). The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. To confirm MIC and to establish minimum bactericidal and fungicidal concentrations (MBC and MFC), 10 µl of each culture medium for bacteria and fungi with no visible growth were removed from each well and inoculated, in triplicates, on nutrient agar and sabouraud dextrose agar plates respectively. After incubation for 24 h at 37°C for bacteria and 3-5 days at 30-35°C for fungi, the number of surviving organisms was determined. MBC and MFC were defined as the lowest extract concentration at which 99.9% of the microorganism was killed. The extract tested in this study was screened three times for each organism.

Scanning electron microscope (SEM) and transmission electron microscope (TEM) observations

Scanning electron microscope observations were carried out on S. aureus (ATCC 29247) and E. coli (ATCC 25922) as an example ofthe extract effect on the bacterial cells. One milliliter of 10^8 CFU/ml of *S.aureus* , *E.coli* bacteria suspension were incubated with extract concentration of 100 and 200 mg/ml, respectively, at 37°C for 24 h. A 10% DMSO-treated culture was used as a control. The control and the extract treated cells were fixed in 4% glutaraldehyde and later postfixed in 1% osmium tetraoxide (0.1M cacodylate buffer, pH 7.4) at room temperature. After eliminating the remaining osmium tetraoxide, the samples were dehydrated in a graduated cold ethanol series (35 to 100%). Each step was performed for about 10 to 15 min at room temperature. The specimens were then dried in the critical point dryer (Baltec, CPD 030). The dried specimens were mounted onto stubs by double-sided carbon tape. The specimens were coated with a thin layer of gold by a Polaron SC 502 sputter coater, and were examined in the Scanning Electron Microscope (Philips, XL30 ESEM). For the transmission electron microscope observation after the dehydration step, the fixed cells were embedded with Epon and the small blocks of bacteria were cut with an ultramicrotome (Reichert Jung Ultracut-S, Leica). The ultra thin sections were then analyzed using TEM (Philips, EM400 HMG).

Statistical analysis

The disc diffusion assay was conducted in triplicates wells in three independent experiments. Therefore, the results were expressed as mean ± SD. SPSS software version 12.0.0.2 was used.

RESULTS

Disc diffusion assay for bacteria

Qualitative and quantitative results were obtained by both inhibition zone and zone diameter (Table 1). The methanol extract of *Vigna radata*, or MBS, showed significant inhibitory effect against the growth of both gram negative and positive bacteria. The inhibition zone for the gram positive bacteria by extract concentration 200 and 500 mg/ml was remarkable ranging from 10 to 16 and 15 to 20 mm respectively. In comparison with the used standard drug, Streptomycin, which gave only 18 mm zone of inhibition. The antibacterial effect of MBS against gram positive bacteria was highly significant. Interestingly, the methanol crude extract of MBS had a significant antibacterial effect against the problematic bacteria MRSA with 7 and 15 mm zone of inhibition at concentrations 200 and 500 mg/ml, respectively versus complete resistance to Streptomycin, the standard drug. This provided evidence that MBS can be cheap and effective anti-MRSA source for both natural and pharmaceutical industry. In contrast, to all previous studies in the field of antibacterial natural products, the *V. mungo* methanol crude extract revealed highly significant antibacterial effect against all the tested Gram negative bacteria. The zone of inhibition ranged from 12 to 16 and 16 to 21 mm at MBS concentrations 200 and 500 mg/ml, respectively. On the other hand, the zone of inhibition of the standard drug, Streptomycin, ranged from 11 to 19 mm only indicating that MBS extract at concentration 500 mg/ml exerted more potent antibacterial effect than Streptomycin did.

Microdilution assay for bacteria

The MIC of the MBS extract against the gram positive bacteria was 100 mg/ml for *Bacillus subtilis* and *S. aureus* while, for MRSA, it was 200 mg/ml. The MBC was 400, 500, and 200 mg/ml for *S. aureus,* MRSA, and *B. subtilis,* respectively (Table 2). On the other hand, the MIC of MBS extract against the tested gram negative bacteria was 200 mg/ml except for *P. aeruginosa* and *Klebsiella pneumoniae* were 300 and 400 mg/ml respectively. The confirmatory test, the MBC, was 500 mg/ml except for *E. coli* O157:H7 and *P. aeruginosa* were 400 mg/ml (Table 2). The decrease in the O.D which reflects the decrease in the bacterial growth was clear and was dose dependent (Figure 1). Accordingly, the findings of the MIC and MBC for the extract of MBS were in conformity with the primary screening test, disc diffusion assay.

This provided more evidence that MBS extract can be a potent source of effective antibacterial compounds against both gram positive and negative bacteria which favors the idea that MBS most likely possesses multiple antibacterial compounds of different mechanism of actions.

Antimicrobial assays for fungi

The methanol crude extract of the MBS showed unprecedented antifungal action against two out of the 12 tested fungi; namely, *T. rubrum* and *T. harzianum* (Table 3). The clear inhibition zone on *T. rubrum* and *T. harzianum* using 700 mg/ml MBS extract, 12 and 15 mm respectively, was greater than that of the standard drug, namely Amphotericin B, 11 and 10 mm, respectively. Given that *T. rubrum* and *T. harzianum* are slow growing and were found usually resistant to most of antifungal drugs, the novel antifungal activity of MBS discovered in the current study might indicate a potent fact-acting antifungal substance. To confirm the antifungal activity of MBS extract, both MIC and MFC were measured. The MIC was 75 mg/ml for both fungi, while MFC was 300 mg/ml for *T. rubrum* and 150 mg/ml for *T. harzianum* (Table 4). The growth inhibition was dose dependent and was reflected by the decrease in O.D. value (Figure 2).

Mode of antimicrobial action

The nature of the antibacterial effect of the extract in regard to inhibition/killing of tested bacteria is important. The MBC: MIC ratio for bacteria or MFC: MIC for fungi is used to specify the nature of the antimicrobial effect

Table 2. The MIC and MBC values against the tested bacteria by the microdilution method.

Bacterial species	Number of strains	MIC* (mg/ml)	MBC† (mg/ml)	MBC: MIC ratio	Antibacterial mode	Positive control Streptomycin µg/ml	
						MIC	MBC
S. aureus	2(clinical isolate)	100	400	4: 1	Bacteristatic	0.065	0.104
S. aureus	1(ATCC 29247 stain)	100	500	5: 1	Bacteristatic	0.06	0.1
MRSA[a]	1(clinical isolate)	200	400	2: 1	Bactericidal	-[b]	-
B. subtilis	1(clinical isolate)	100	200	2: 1	Bactericidal	0.04	0.08
E. coli	1(clinical isolate)	200	500	2.5: 1	Borderline Bacteristatic	0.05	0.1
E. coli	1(ATCC 25922 strain)	200	500	2.5: 1	Borderline Bacteristatic	0.045	0.09
E. coli	1(ATCC 35218 strain)	200	500	2.5: 1	Borderline Bacteristatic	0.042	0.08
E. coli O157:H7	1(clinical isolate)	200	400	2: 1	Bactericidal	0.049	0.09
P. aeruginosa	1(clinical isolate)	300	400	1.33: 1	Bactericidal	0.047	0.08
K. pneumoniae	1(clinical isolate)	400	500	1.25: 1	Bactericidal	0.05	0.085
S. Typhimurium	1(ATCC 25241 strain)	200	400	2: 1	Bactericidal	0.048	0.082

*minimum inhibitory concentration, †: minimum bactericidal concentration, [a] : Methicillin-resistant *S. aureus*, [b] : (-) means no growth inhibition detected.

Table 3. Antifungal activity of mung bean sprout methanol extract by disc diffusion method.

Microorganisms		Inhibition zone in diameter (mm)				
Fungal species	Number of strains	*B. oleracea* methanol extract (mg/ml)			Negative control (MeOH*)	Positive control Amphotericin B (10µg/disc)
		200	500	700		
Trichophyton rubrum	1 (ATCC 11990)	-	-	12 ± 1.3	- †	11 ± 0.6
Microsporum canis	1 (ATCC 8137)	-	-	-	-	10 ± 0.6
Aspergillus niger	1 isolate	-	-	-	-	18 ± 1.0
A. terreus	1 (ATCC 20542)	-	-	-	-	10 ± 0.8
A. oryzae	1 isolate	-	-	-	-	8 ± 0.8
Paecilomyces variatii	1 isolate	-	-	-	-	18 ± 1.3
Phanerochaete crysosporium	1 isolate	-	-	-	-	19 ± 1.3
Trichoderma sp.	1 isolate	-	-	-	-	-
Trichoderma harzianum	1 (ATCC 20671)	-	-	15 ± 0.6	-	10 ± 0.8
Trichoderma atroviride	1 (ATCC 74058)	-	-	-	-	-

*MeOH: methanol. †: (-) means no growth inhibition zone.

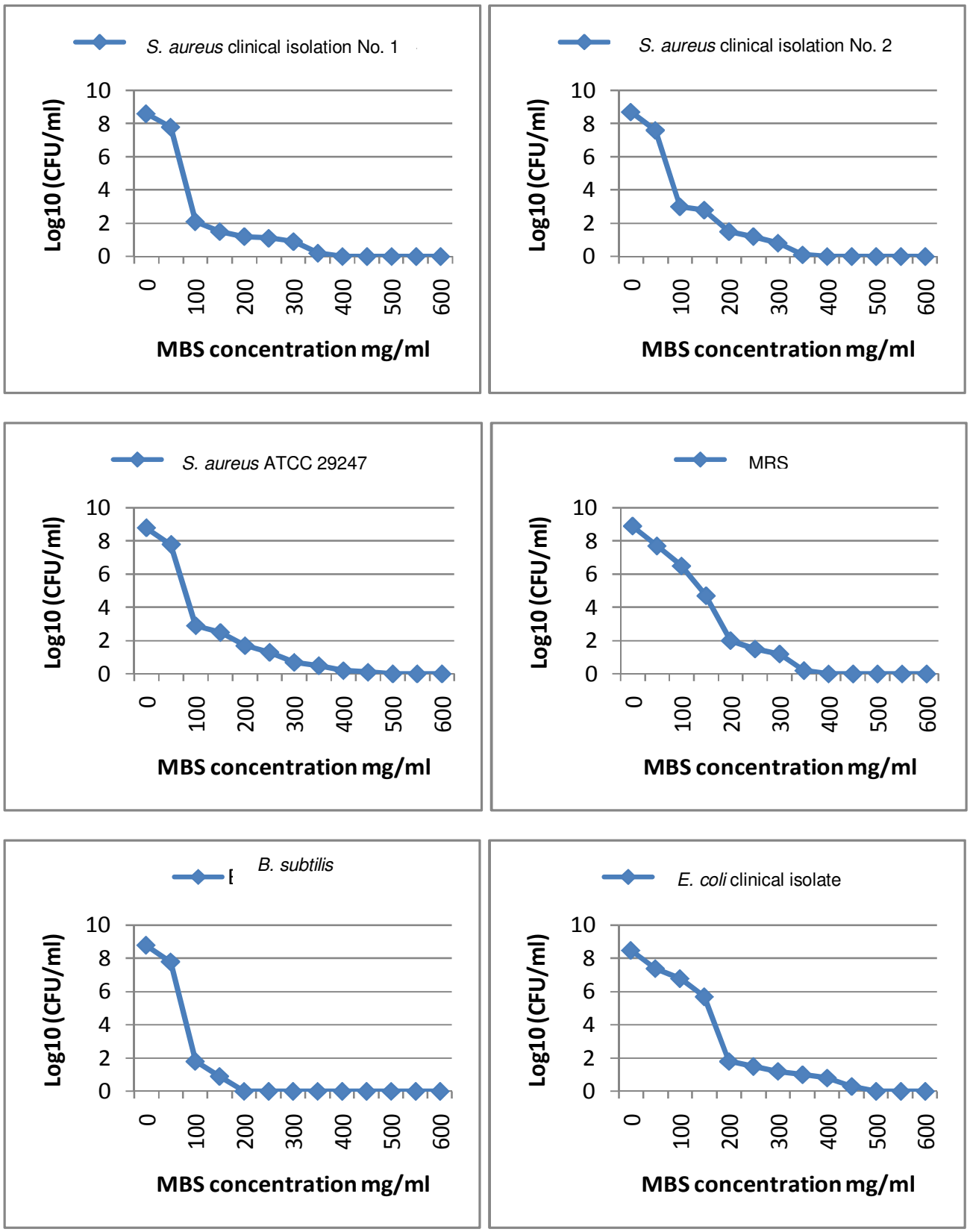

Figure 1. Diagrams reflect the decrease in Gram positive and Gram negative bacterial growth which demonstrated by log10 measured by microdilution assay. The decrease in bacterial growth resulted from MBS methanol crude extract treatment in different concentrations.

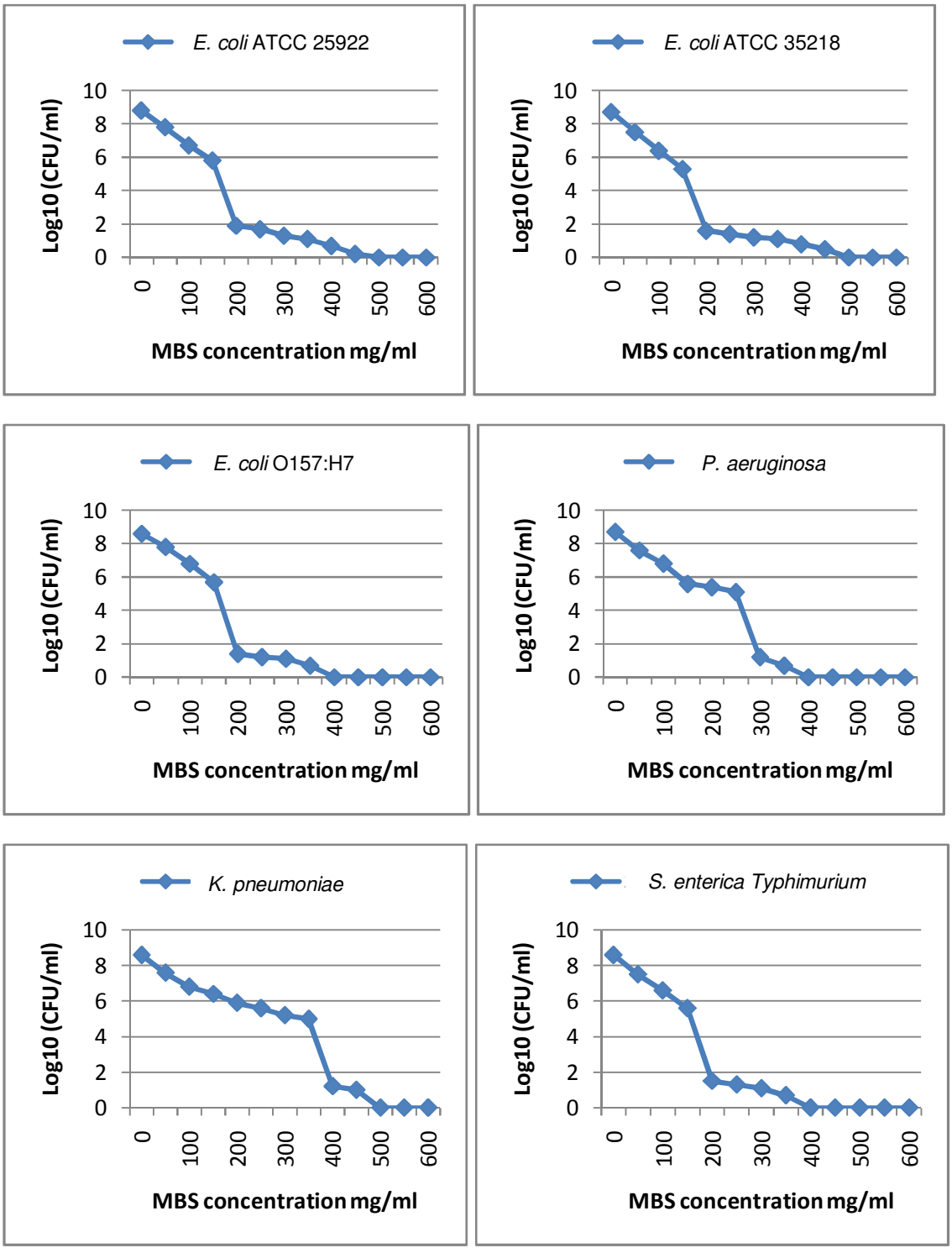

Figure 1. Contd.

against any given pathogen (Barry et al., 1999). When the MBC: MIC or MFC: MIC ratio of a pathogen is between 1:1 to 2:1, the chemical substance is considered as bactericidal or fungicidal against that pathogen (Barry et al., 1999). On the other hand, if ratio was > 2:1, the mode of antimicrobial action is more likely to be bacteristatic or fungistatic. Therefore, MBC: MIC or MFC: MIC ratio was calculated for each bacterial or fungal

Table 4. The MIC and MFC values against the tested fungi by the microdilution method.

Fungal species	Number of strains	MIC* (mg/ml)	MFC† (mg/ml)	MFC: MIC ratio	Antifungal mode	Positive control Amphotericin B µg/ml	
						MIC	MBC
T. rubrum	1 (ATCC 11990)	75	300	4: 1	Fungistatic	2	3
T. harzianum	1 (ATCC strain 20671)	75	150	2: 1	Fungicidal	2.5	2.9

*minimum inhibitory concentration, †: minimum fungicidal concentration.

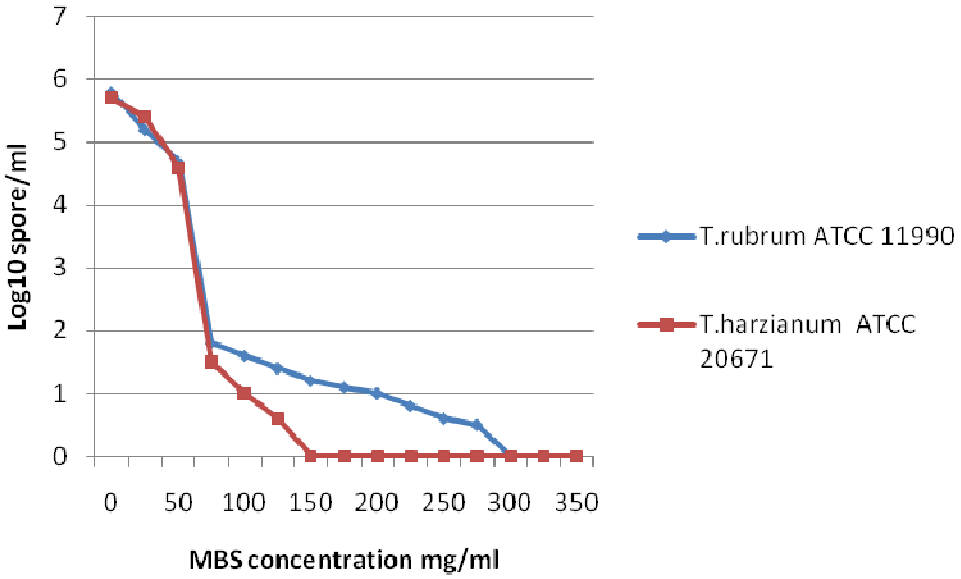

Figure 2. The diagram demonstrates the growth inhibtion of the sensitive fungi expressed by log10 which detected by the microdilution assay after incubation with MBS methanol crude extract in different concentrations.

Pathogen, respectively. It was found that the tested extract of MBS exerted a clear bactericidal effect against 2 gram positive bacteria namely, MRSA and *B. subtilis* and 4 gram negative bacteria namely *E. coli* O157:H7, *P. aeruginosa*, *K. pneumoniae*, and *S.* Typhymurium. Besides, MBS extract exerted borderline bactericidal effect on 3 non-O157:H7 *E. coli* bacteria. On the other hand, bacteristatic effect of MBS was seen only for non-MRSA *S. aureus* bacteria (Table 2). For fungi, the extract exerted fungicidal effect against *T. harzianum* and fungistatic against *T. rubrum* (Table 4).

SEM and TEM electron microscope observations

The antibacterial effect of MBS extract was seen by SEM on *S. aureus, E. coli*. After exposing *S. aureus* to 100 mg/ml and *E. coli* to 200 mg/ml of the methanol extract, the cells were collapsed, perforated and become sticky, almost seen like a shrunken cells as compared to control (Figure 3). While the antibacterial effect which had been observed by TEM was demonstrated as clear morphorlogical changes in the treated cells, in addition to the detachment of the cell membrane (Figure 4).

DISCUSSION

The wide spread of drug resistant microbe raises the need for new, cheap, effective, and safe drugs. One of the best candidates to address this need appears to be the natural sources. Nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds. Therefore, they are most often obtained through initial ethanol or methanol extraction (Ahmad et al., 1998; Cowan, 1999; Eloff, 1998; Lin et al., 1999). Therefore, we used methanol as a solvent to extract almost all of the proposed antimicrobial agents in order to prepare the basis for monitoring different antimicrobial agents as a prelude for the future separation of single antimicrobial compound(s). One of the interesting points regarding

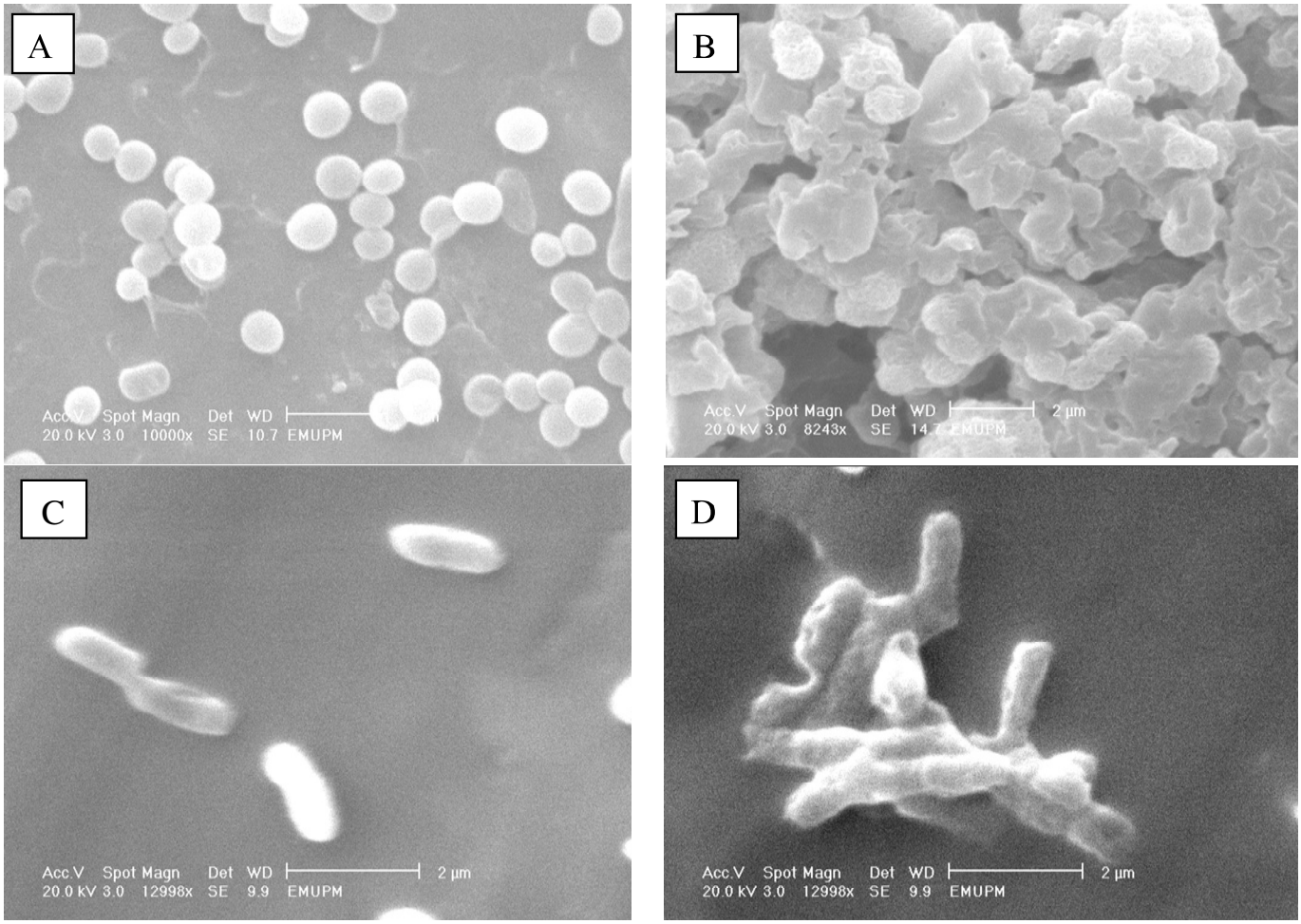

Figure 3. The scanning electron microscope study of MBS methanol extract showing the cytological changes in *S. aureus* cells: (A) control cells (B) treated cells and in *E. coli* cells: (C) control cells (D) treated cells.

MBS extract, its pH range, 6.8 to 7.0, is neutral which eliminates the possibility that the antimicrobial effect of MBS is attributed to its ability to create acidic environment. It was proposed that the possible mechanism of antimicrobial activity by some of the phenolic containing extracts is by creating an acidic environment that causes the bacterial cell membrane disruption (Randhir and Shetty, 2007).

Seed sprouts have long been used in the diet as health food and recent research shows that in addition to being a good source of basic nutrients, they also have important phytochemicals with disease preventive and health promoting properties (Kurtzweil, 1999). Moreover, germinating seeds, or sprouts, are believed to have stronger defenses and metabolic pathways than the parent seeds (Fernandez-Orozco et al., 2008; Mwikya et al., 2001). This study assumed that one of the enhanced defense mechanisms and modified phytochemical activities in sprouts might be the synthesis of competent antimicrobial phytochemicals that might share their antimicrobial effect with human and animal pathogens. The findings of the current study backed up strongly such assumption. The current study unveiled a novel, powerful, and broad spectrum antimicrobial activity of MBS against a large number of human and animal pathogenic microbes including MDR bacteria, non- MDR bacteria, and fungi. On the other hand, methanol extract of Mung beans (not MBS) was subjected to the same experiment done for MBS. Unlike MBS extract, Mung bean extract showed no remarkable antimicrobial activity against all tested bacteria and fungi [data not shown]. This provided stronger evidence that MBS chemical structure is much different from that of Mung beans and the germination of Mung beans into MBS resulted to synthesis and/or modification of the phytochemical structure leading to formation of new antimicrobial active compounds. Previous studies have isolated a combination of antimicrobial protein from mung bean which appeared to be active against a range of bacteria and fungi (Lin et al., 2007; Wang et al., 2004a, b, 2005,

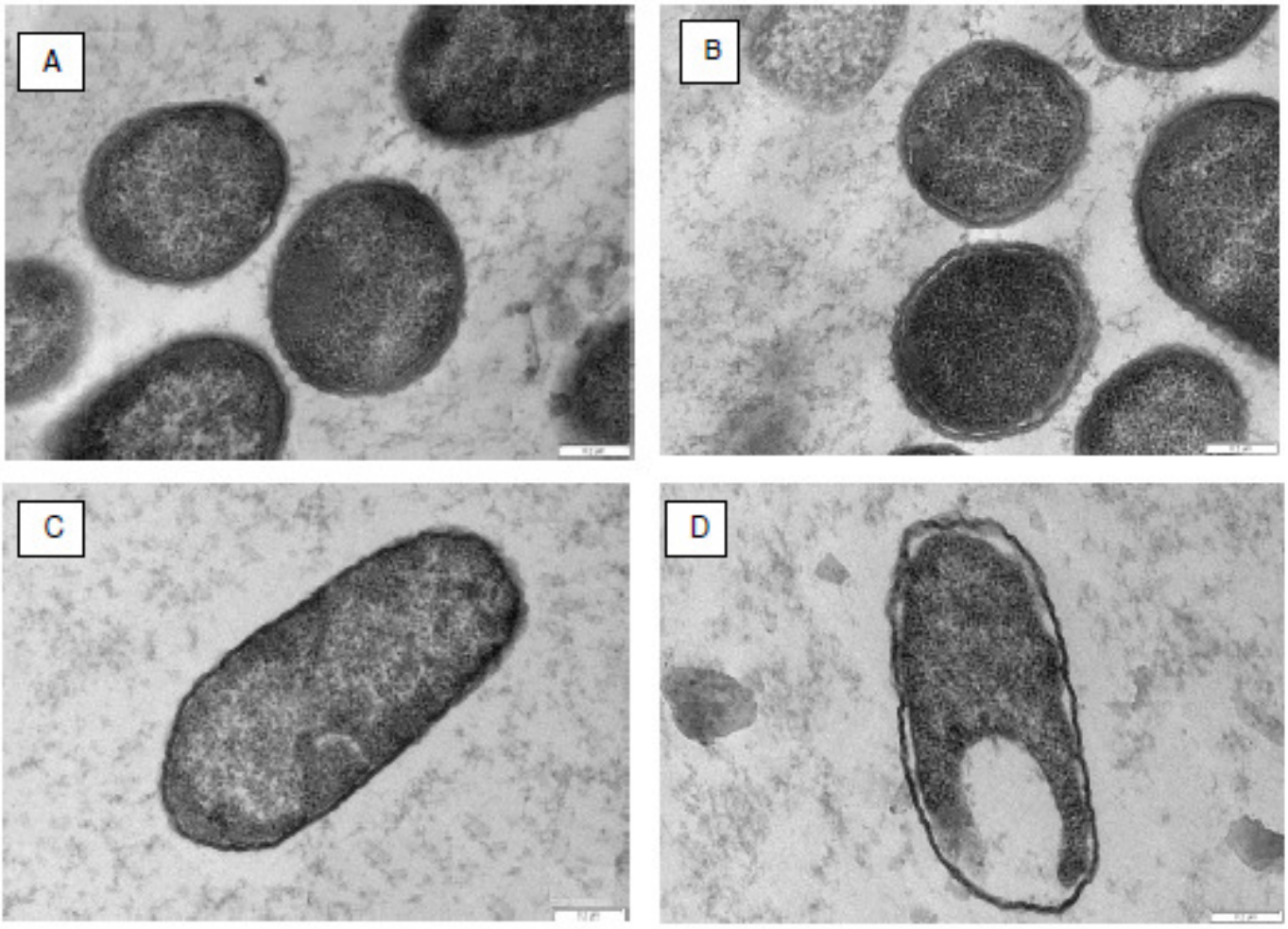

Figure 4. The transmission electron microscope study of MBS methanol extract showing the cytological changes in *S. aureus* cells: (A) control cells: magnification 60.000k (B) treated cells: magnification 60.000k and in *E. coli* cells: (C) control cells: magnification 60.000k (D) treated cells: magnification 60.000k.

2006; Ye and Ng, 2005). However, few or no studies have focused on the phytochemical profile of the mung bean or mung bean sprout, such as the current methanol extract of MBS, as antimicrobial agents. A study found that sprouting improved the antioxidant activity due to the higher demand for oxygen during early stages of the germination; therefore, phenolics might be protecting the cells from potential oxidation-induced deterioration (Lambert, 2008). Another study confirmed the presence of six flavonoids, that is, robinin, rutin, kaempferol, quercetin, isoquercitrin, and kaempferol-7-O-rhamnoside, and found that the content of these flavonoids increased during the germination of mung beans (Sawa et al., 1999). Therefore, the increased antioxidant activity found in MBS together with the potent type of flavonoids might explain the significant antibacterial activity against the tested bacteria.

Unfortunately, the antimicrobial potential of MBS extracts against gram negative and positive bacteria has long been underestimated and not fully documented. The two previous reports found studying the antimicrobial activity of MBS were conducted on one bacterium only, *Helicobacter pylori*, that causes gastroduodenal disease (Mitchell and Megraud, 2002; Randhir et al., 2004). Surprisingly, no other studies were conducted on other bacteria or fungi. Therefore, we believe that the findings of the current study might be a breakthrough in the field of microbiology, antimicrobials, and natural products. MBS crude extract yielded a very promising antimicrobial activity against 11 bacteria including 3 highly MDR bacteria; MRSA, or a bit VISA, P. aeruginosa, and E. coli O157:H7. The most interesting point is that, MBS exerted bactericidal rather than bacteristatic effect on these 3 MDR bacteria and, at disc diffusion assay, resulted in clear inhibition zones equal or greater than the standard drug, streptomycin.

Hospitals across the United States and around the world are fighting the escalation of drug-resistant

infections (Bonuel et al., 2009). For MRSA isolate, a considerable inhibition zone of bactericidal nature, MBC: MIC ratio is 2:1, was driven by the MBS extract at concentration 500 mg/ml, 17 mm versus 0 mm of standard drug. The effect of MBS on MRSA was outstanding when compared with the bacteristatic effect of MBS on *S. aureus*, 20 mm versus 18 mm of standard drug. The mechanism of action for the predilection of MBS antibacterial effect to MRSA is still not explained. Taken into account that the tested MRSA was found of intermediate response to vancomycin (VISA), the antibacterial agent in MBS responsible for this action must be a top priority to be studied and discovered. It is worth mentioning that MRSA –VISA strains present a thickening of the cell wall which is believed to deplete the vancomycin available to kill the bacteria which worries many physicians and microbiologists in regard to near future prospects of therapy (Sng et al., 2005).

Accordingly, the current MBS anti-MRSA activity and its underlying mechanism of action seem novel and different from other known antibiotics. This might combat the increase in community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections that have been reported in many literatures (Creel et al., 2009). Another problem facing international health bodies worldwide, the EHEC *E. coli* O157:H7- driven diarrheal disease which is a major cause of illness and death among infants and young children worldwide (Dedeic-Ljubovic et al., 2009). Recently, *E. coli* O157:H7 was shown to be highly resistant to different antibiotics, all commonly used, by clinicians and veterinarians, for the treatment of infections with gram-negative bacteria (Saleh et al., 2009). For this reason, the used isolate of EHEC O157:H7 in the current study was selected to be highly resistant isolate against four antibiotics. In the current study, the antibacterial activity of MBS extract against *E.coli* O157:H7 was remarkable. The inhibition zone was about 17 mm at concentration 500 mg/ml of the extract which is only 1 mm less than the diameter of the standard antibiotic, streptomycin. Moreover, the MBC: MIC ratio was 2:1 revealing potent bactericidal effect against this notorious bug. Therefore, the extract of MBS can also be considered as a source of a new candidate antibacterial agent for both human and animal *E. coli* O157:H7 diseases. *P. aeruginosa* is an opportunistic pathogen and it is a serious problem in patients hospitalized with cancer, cystic fibrosis, and burns. And the case fatality rate in these patients is near 50% (Giamarellou and Kanellakopoulou, 2008). *P. aeruginosa* is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics (Taccetti et al., 2008). The isolate of *P. aeruginosa* used in the current study was selected to be highly resistant against 6 different antibiotics. Nevertheless, the MBS extract of the current study showed 19 mm inhibition zone which was 1 mm larger than that of streptomycin itself, 18 mm. Moreover, MBC (400): MIC (300) mg/ml, 1.33:1 ratio, was the lowest among all other studied bacteria and indicated that MBS extract exerted a very potent bactericidal effect against one of the most highly resistant isolates of *P. aeruginosa*. Interestingly, like the isolates of MRSA and *E. coli* O157:H7, the antibacterial activity of MBS extract against *P. aeruginosa* seems different from all the antibiotics that *P. aeruginosa* is resistant to.

Collectively, three used MDR bacteria were resistant to 15 different powerful antibiotics belonging to 8 different groups of antibiotics. These 8 groups belong to 5 common mode of actions included cell wall targeting penicillin and cephalosporin's 3rd and 4th generations, 30S ribosomal subunit targeting glycopeptides and polyketide, 50S ribosomal subunit targeting lincosamide and macrolides, DNA synthesis inhibitors by fluoroquinolones, and sulfonamides which are enzymes competitors. Since, MBS extract exerted powerful bactericidal effect against these 3 highly MDR bacterial isolates, the antimicrobial effect of MBS seems to bypass the most common pathways of antibiotics resistance. Therefore, this gave a clue on the high possibility for the presence of novel antimicrobial compound(s) in MBS extract that might not share the same mode of action with the above mentioned antibiotics or at least have some modified interactions of the same pathway. For fungi, although *T. harzianum* is a rare opportunistic pathogen, it had been detected as a disseminated fungal infection in the postmortem examination of a renal transplant recipient. The case illustrates the widening spectrum of opportunistic *Trichoderma* spp. in immunocompromised patients (Guarro et al., 1999).

Recently, it was isolated from blood serum, skin lesions, sputum, and throat of a pediatric patient with neutropenia (Kantarciolu et al., 2009). On the other hand, *T. rubrum* is the most common dermatophyte species and the most frequent cause of fungal skin infections in humans worldwide. It's a major concern because feet and nail infections caused by this organism is extremely difficult to cure (Yang et al., 2007). In addition, it is resistant to the most of the commercially available antifungal agents that had been recorded (Mukherjee et al., 2003). The effective antifungal activities obtained by using MBS extract against *T. harzianum* and *T. rubrum* were as remarkable as these for bacteria. The reason behind, the antifungal activity of MBS was compared with one of the most powerful antifungal drugs, namely amphotericin B. The inhibition zones of MBS for *T. rubrum,* 12 mm, and *T. harzianum*, 15 mm, were higher than that of amphotericin B, 11 and 10 mm respectively. However, the mechanism of action is not yet explained which needs, in the future, to separate and test the single antifungal compounds present in MBS.

There are many possibilities for the effective antimicrobial activities gained in the results of the current study. It is known that flavonoids are substances which are synthesized by plants in response to microbial infection (Dixon et al., 1983), it should not be surprising

that they have been found *in-vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Tsuchiya et al., 1996) and may be with fungal cell wall too. Modes of action reported for flavonoids are, lysis and leakage of intracellular constituents, perturbation of cell homeostasis, inhibition of enzymes, electron transport, oxidative phosphorylation, interaction with macromolecules and effects on macromolecular biosynthetic processes (Lambert, 2008).

Many studies tried to investigate the crude plant extract effect on bacteria to support the antibacterial activity they gained from the disc diffusion and the microdilution assays. In this study the electron microscopic observations for both SEM and TEM demonstrated the effect of the methanol extract on bacterial cells, namely, *S. aureus* and *E. coli*. There were clear morphological changes with shrunken and collapsed form in the teated bacteria. This could be due to the leak in the cell wall or perhaps some alteration in the membrane permeability. These results strengthen our achievements to consider the crude methanol extract of MBS as promising antimicrobial agent.

Conclusion

Taken together, it was concluded that the MBS extract using acid methanol solvent has novel and potent antimicrobial effect against the tested gram negative, gram positive bacteria, and fungi while the mung bean extract did not show similar potent antimicrobial activity. These findings support the hypothesis of the enhanced and modified phytochemicals synthesis and defense mechanisms in sprouting seeds. Furthermore, the antimicrobial activity of MBS was shown to be not simply attributed to creating acidic environment. Most interestingly, for the first time, MBS extract showed potent bactericidal effect against three highly resistant MDR bacterial isolates which are, MRSA, *E. coli* O157:H7, and *P. aeroginusa.* In addition, other tested bacteria were shown to be sensitive to MBS antimicrobial activity like *S.* Typhimurium, *K. pneumonae, S. aureus*, non-EHEC *E. coli*, and *B. subtilis.* Moreover, MBS extract showed potent antifungal activity against *T. rubrum* and *T. harzianum*. The wide range of antimicrobial activities of MBS against both MDR and non MDR microbes points out to the possibility of the presence of more than one novel antimicrobial component differing largely in their mode of action from the current antibiotics to which the tested MDR bacteria were resistant.

Further investigations on the active antimicrobial components in the MBS methanol crude extract are necessarily required to provide the pharmaceutical companies with cheap, effective, and most likely novel single antimicrobial agent(s) active against gram positive and negative bacteria as well as fungi.

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Abbreviations: MBS, mung beans sprout; **MDR**, multiple drug resistant; **MRSA**, methicillin-resistant *Staphylococcus aureus*; **EHEC**, Enterohemorrhagic *Escherichia coli*; **VISA**, vancomycin-intermediate *Staphylococcus aureus*; **MIC**, minimum inhibitory concentration; **MBC**, minimum bactericidal concentration; **MFC**, minimum fungicidal concentration; **SEM**, scanning electron microscope; **TEM**, transmission electron microscope.

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