การวิเคราะห์พฤกษเคมีและผลของแฟรกชั่นจากสารสกัดใบชุมเห็ดเทศต่อแบคทีเรีย Methicillin Resistant *Staphylococcus Aureus* และ *Pseudomonas Aeruginosa*

Phytochemical Analysis and Effect of *Senna Alata* Leaf Extract Fractions on Methicillin Resistant *Staphylococcus Aureus* and *Pseudomonas Aeruginosa*

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บทคัดย่อ

การอุบัติของแบคทีเรียที่ดื้อยาปฏิชีวนะหลายชนิด ทำให้มีความจำเป็นที่ต้องพัฒนายาต้านแบคทีเรียชนิดใหม่ขึ้นมา พืชสมุนไพรถือเป็นอีกหนึ่งตัวเลือกที่ถูกนำมาใช้ในการยับยั้งการเจริญของเชื้อแบคทีเรีย เนื่องจากพืชสมุนไพรมีความเป็นพิษน้อยและหาได้ง่ายตามธรรมชาติ ดังนั้นการศึกษาในครั้งนี้คณะผู้วิจัยมีวัตถุประสงค์เพื่อทดสอบผลของแฟรกชั่นจากสารสกัดใบชุมเห็ดเทศ *(Senna alata)* ในการยับยั้งการเจริญและผลต่อรูปร่างของ methicillin-resistant *Staphylococcus aureus* (MRSA) และ *Pseudomonas aeruginosa* โดยสารสกัดหยาบจากใบชุมเห็ดเทศได้ถูกนำมาสกัดแฟรกชั่นด้วยวิธี คอลัมน์โครมาโตกราฟี จากนั้นนำไปทดสอบหาความเข้มข้นต่ำสุดในการยับยั้ง (minimum inhibitory concentration, MIC) และฆ่าเชื้อแบคทีเรีย (minimum bactericidal concentration, MBC) ซึ่งพบว่าจากแฟรกชั่นทั้งหมด 13 ส่วน แฟรกชั่นที่ 13 (F13) มีประสิทธิภาพสูงสุดในการยับยั้ง MRSA ที่ MIC 0.39±0.00 มก./มล. และ MBC 5.21±1.80 มก./มล. และ แฟรกชั่นที่ 3 (F3) มีประสิทธิภาพสูงสุดในการยับยั้ง *P. aeruginosa* ที่ MIC 0.78±0.00 มก./มล. และ MBC 5.21±1.80 มก./มล. เมื่อนำแฟรกชั่น F3 และ F13 มาทำการตรวจวิเคราะห์ทางด้านพฤกษเคมีจะพบฟลาโวนอยด์, ฟินอลิก และแทนนินในแฟรกชั่นทั้งสองส่วน ในขณะที่อัลคาลอยด์จะพบเฉพาะในแฟรกชั่น F3 และสเตอรอยด์จะพบเฉพาะในแฟรกชั่น F13 เท่านั้น และเมื่อนำแฟรกชั่น F3 และ F13 มาทดสอบผลที่มีต่อรูปร่างของเชื้อแบคทีเรียด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่าใน MRSA แฟรกชั่น F3 มีผลทำให้เกิดการแตกของเซลล์ และแฟรกชั่น F13 ส่งผลให้ผิวเซลล์มีลักษณะขรุขระ ในขณะที่ *P. aeruginosa* นั้น แฟรกชั่น F3 จะทำให้เซลล์มีลักษณะบุ๋มบริเวณตรงกลางเซลล์ และแฟรกชั่น F13 จะส่งผลให้มีการยืดตัวของเซลล์และผิวเซลล์ไม่เรียบ ดังนั้นจากผลการวิจัยในครั้งนี้แสดงให้เห็นว่าแฟรกชั่นของสารสกัดจากใบชุมเห็ดเทศมีผลในการยับยั้งการเจริญและทำให้เกิดการเปลี่ยนแปลงรูปร่างของเซลล์ที่แตกต่างกันในแบคทีเรียแกรมบวก MRSA และแกรมลบ *P. aeruginosa*

คำสำคัญ : ชุมเห็ดเทศ แฟรกชั่น ความเข้มข้นต่ำสุดในการยับยั้งแบคทีเรีย ความเข้มข้นต่ำสุดในการฆ่าเชื้อแบคทีเรีย MRSA *Pseudomonas aeruginosa*

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Abstract

The emergence of multiple drug resistance (MDR) bacteria necessitates the development of new antimicrobial agents. Medicinal plants represent potential means of treating infection because they have low toxicity and are readily accessible. The objective of this study is to determine the effect of *Senna alata* fractions on the growth inhibition and cell morphology of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa (P. aeruginosa).* Ethanolic *S. alata* leaf extract was fractionated by column chromatography. The fractions were evaluated for antibacterial activity using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, and their effects on bacterial cell morphology were investigated by scanning electron microscopy (SEM). From 13 fractions of *S. alata* extract, activity against MRSA was most pronounced in fraction F13, with MIC and MBC values of 0.39±0.00 mg/ml and 5.21±1.80 mg/ml, respectively. Activity against *P. aeruginosa* was most pronounced in fraction F3 with MIC and MBC values of 0.78±0.00 mg/ml and 5.21±1.80 mg/ml, respectively. Phytochemical analysis of fractions F3 and F13 revealed the presence of flavonoids, phenolics and tannins in both fractions, whereas alkaloids and steroids were found only in F3 and F13, respectively. SEM examination revealed membrane shrinkage in F3-treated MRSA and bursting cells with a crystalline rough surface in F13-treated MRSA. *P. aeruginosa* cells showed a small rupture at their center following treatment with fraction F3, while surface roughening and elongated cell shape were observed following treatment with fraction F13. These results demonstrate that different *S. alata* fractions exhibit different effects on cell morphology and have different levels of inhibitory and bactericidal activity against Gram-positive, MRSA, and Gram-negative, *P. aeruginosa.*

Keywords: *Senna alata*, fractions, minimum inhibitory concentration, minimum bactericidal concentration, MRSA, *Pseudomonas aeruginosa*

Introcuction

Infectious diseases are an important public health problem, one that has been rendered more complicated by the emergence of multidrug resistant (MDR) bacteria. Multidrug resistance is especially remarkable in Asia because of a lack of awareness and understanding of appropriate antimicrobial usage and different standards of public hygiene between countries.[1,2] Two outstanding examples are methicillin-resistant *Staphylococcus aureus* (MRSA) and MDR *Pseudomonas aeruginosa*. These pathogens cause infections that are difficult to control in terms of both treatment and prevention of dissemination.[3-5] The emergence of these MDR bacterial strains necessitates the development of new antimicrobial agents. Medicinal plants represent potential means of treating infection because they have low toxicity and are readily accessible.[6] *Senna alata* (Linn.) Roxb. from the family Fabaceae (Leguminosae) is commonly known as ringworm tree, candlestick, candle bush or *chum-het-thet*, and is often used as a traditional medicine in Thailand. Pharmacological and biological activities of *S. alata* reportedly include laxative effects, anti-inflammatatory activity, pain relief, increased urination, perspiration, the promotion of digestion, hypoglycemic activity, and insect repellent activity.[7,8] Previous studies have also shown that *S. alata* crude extract has antibacterial and antifungal activity,[9-14] but the active components have yet to be identified.

In the present study, *S. alata* leaves were extracted using ethanol in accordance with Thai traditional medicine practice. The crude extract was then fractionated by column chromatography, with the fractions examined for antibacterial activities. The effect of active fractions on the cell morphology of Gram-positive MRSA and Gram-negative *P. aeruginosa* was also determined to gain deeper understanding of how the active components affect bacterial cells.

Materials and Methods

Plant material

S. alata leaves were purchased from a traditional

herbal pharmacy in Maha Sarakham province, Thailand. The leaves were cleaned and air-dried before drying in a hot air oven at 50 °C for 5 hours. The dried leaves were then ground into a fine powder using an electric blender. This powder was stored in airtight containers and kept at room temperature until used.[11,12]

Plant extraction and fractionation

Ten grams of powdered *S. alata* leaves was extracted with 1 L of 95% ethanol using Soxhlet apparatus at 60-70 °C for 3 hours. The ethanol from the crude extract was completely removed using a rotary evaporator. The crude extract was then fractioned by separation column chromatography using dichloromethane and ethanol as the solvents. At first, 100% dichloromethane was used as the solvent, then steadily decreased the proportion of dichloromethane and increased the proportion of ethanol until the solvent was 100% ethanol. Elutes were collected and aliquots subjected to Thin layer chromatography (TLC) for fractions analysis under UV light at 254 and 365 nm. Fractions which showed the same pattern on TLC were pooled together and then subjected to evaporation in a rotary evaporator and freeze-dried in a lyophilizer.[12,14] Dried fractions were dissolved in 50% DMSO to make a stock concentration of 100 mg/ml and were stored at 4°C for further use.[15]

Preparation of bacterial inocula

MRSA (DMST 20651) and *P. aeruginosa* (ATCC 27853) were cultured at 37 °C for 18-24 hours on nutrient agar (NA). The bacteria were subsequently subcultured in Mueller-Hinton broth (MHB), incubated at 37 °C for 3 hours and adjusted to 0.5 McFarland standards. Then, the bacterial suspension was further diluted, giving approximately 10^6 CFU/ml.[6]

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *S. alata* fractions

MICs were determined by performing the broth microdilution method in 96-well plates. A 100 µl volume of each *S. alata* fraction was pipetted into the first row of the plate. To all other wells, 50 µl of MHB was added and mixed with 50 µl of each fraction in sequence for two-fold serial dilutions. Then, 50 µl of adjusted bacterial suspension was put into each well. The 96-well plates were incubated for 16-18 hours at 37 °C. After that, 10 µl of 1 mg/ml of resazurin indicator solution was added to each well and incubated for 2 hours at 37 °C. If a color change from purple to pink or colorless was detected, this was recorded as positive.[15] The lowest concentration at which the color change occurred was recorded as the MIC. DMSO was used as a viable cell control, tetracycline was used as a positive control, and MHB was used as a negative control. All experiments were performed in triplicate.

To determine the MBC, a loopful of suspension from all the wells that did not show any change in color were inoculated on NA and incubated for 24 hours at 37 °C. After incubation, the lowest concentration with no visible growth was recorded as the MBC.[16]

Activity index of fractions

The activity index was calculated by dividing the MIC value of each fraction by the MIC value of the crude extract.[17,18]

Phytochemical analysis

The fractions of *S. alata* which showed the lowest MIC were subjected to standard qualitative phytochemical analysis for different secondary metabolites including alkaloids, flavonoids, phenolics, steroids, and tannins using previously described methods.[16,19] Secondary metabolites that were present in both fractions (i.e. flavonoids, phenolics and tannins) were then analyzed quantitatively. The total flavonoid and total phenolic content were determined using the Folin–Ciocalteu method.[20] Determination of the percent contribution of tannin was performed using the principle that polymeric tannin pigments are resistant to bisulfite bleaching as previously described.[21,22]

Examination of treated bacteria by scanning electron microscopy (SEM)

MRSA and *P. aeruginosa* were cultured in MHB

at 37 °C for 3 hours, adjusted to 0.5 McFarland standards, and inoculated into the 2 x MBC of fractions F3 and F13. These suspensions were incubated for 12 hours at 37 °C. Twenty microliters of each suspension was applied to nuclear pore polycarbonate membranes, and fixed in 2.5% glutaraldehyde at 4 °C for 12 hours. Bacterial cells dehydration process was achieved by increasing concentrations of ethanol (from 30%, 50%, 70%, 80%, up to 100%). The dried samples were mounted onto stubs, coated with 40-60 nm of gold and then observed by SEM (LEO 1450 VP Scanning Electron Microscope).[23]

Results

Extraction and antibacterial activity of the *S. alata* fractions against MRSA and *P. aeruginosa*

Yields of antibacterial activity of the 13 fractions of *S. alata* are presented in Table 1. The crude extract and the 13 fractions were initially tested for antibacterial activity using an agar diffusion method. Only 5 fractions (i.e. F3, F4, F7, F12 and F13) had significant antibacterial activity compared to tetracycline (data not shown). Subsequently, MICs and MBCs of the crude extract and 13 fractions were determined against MRSA and *P. aeruginosa* (Table 2). MIC and MBC values of the fractions against MRSA ranged from 0.39±0.00 to 12.50±0.00 mg/ml and from 5.21±1.80 to 25.00±0.00 mg/ml, respectively. For *P. aeruginosa*, MIC and MBC were from 0.78±0.00 to 25.00±0.00 mg/ml and from 5.21±1.80 to 33.33±14.43 mg/ml, respectively. Of all the 13 fractions, F13 showed the greatest activity against MRSA with MIC and MBC values of 0.39±0.00 mg/ml and 5.21±1.80 mg/ml, whereas F3 showed the greatest activity against *P. aeruginosa* with MIC and MBC values of 0.78±0.00 mg/ml and 5.21±1.80 mg/ml (Table 2).

Table 1 Yields of *S. alata* fractions

Fraction	Yield (%)
F1	11.64
F2	5.45
F3	18.18
F4	10.55
F5	28.73
F6	7.27
F7	9.45
F8	8.73
F9	10.91
F10	10.18
F11	9.82
F12	16.73
F13	13.82

Activity index values of the *S. alata* fractions with reference to the crude extract are shown in Table 2. Eight of the 13 fractions showed better inhibition of MRSA, and five of the 13 fractions showed better inhibition of *P. aeruginosa* than the crude extract. Fraction F13 had the highest activity index against MRSA (21 times better than crude extract), whereas fraction F3 had the highest activity index against *P. aeruginosa* (10.5 times better than crude extract).

Table 2 Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *S. alata* fractions

S. alata fraction	MRSA			*P. aeruginosa*		
	MIC (mg/ml)	MBC (mg/ml)	Activity index	MIC (mg/ml)	MBC (mg/ml)	Activity index
F1	12.50±0.00	25.00±0.00	0.66	25.00±0.00	25.00±0.00	0.33
F2	NT	NT	NT	NT	NT	NT
F3	0.78±0.00	14.58±9.55	10.5	**0.78±0.00**	**5.21±1.80**	**10.5**
F4	0.78±0.00	12.50±0.00	10.5	2.61±0.91	8.33±3.61	3.14
F5	12.50±0.00	33.33±14.43	0.66	25.00±0.00	25.00±0.00	0.33
F6	NT	NT	NT	25.00±0.00	25.00±0.00	0.33
F7	3.13±0.00	20.83±7.22	2.62	10.42±3.61	12.50±0.00	0.79
F8	7.29±4.77	20.83±7.22	1.21	6.25±0.00	12.5.±0.00	1.31
F9	8.33±3.61	12.50±0.00	0.98	6.25±0.00	16.67±7.22	1.31
F10	8.33±3.61	20.83±7.22	0.98	25.00±0.00	25.00±0.00	0.33
F11	NT	NT	NT	25.00±0.00	33.33±14.43	0.25
F12	2.08±0.91	14.58±9.55	3.94	20.83±7.22	33.33±14.43	0.25
F13	0.39±0.00	5.21±1.80	**21**	8.33±3.61	8.33±3.61	3.14
Crude	8.19±0.00	16.38±0.00	N/A	8.19±0.00	8.19±0.00	N/A
Tetracycline	0.016	0.016	N/A	0.004	0.004	N/A

Data were expressed as mean±SD; NT: not tested; N/A: not applicable

Phytochemical analysis of the *S. alata* fractions

S. alata fractions F3 and F13, which had shown the lowest MIC values against MRSA and *P. aeruginosa*, respectively, were screened for the presence of different classes of secondary metabolites.

The results revealed the presence of flavonoids, phenolics and tannins in both fractions (Table 3). In addition, alkaloids were found in fraction F3, and steroids were found in fraction F13.

Quantitative analyses of flavonoid, phenolic and tannin content in the two fractions are shown in Table 4. Fraction F3 had the highest content of total flavonoids (4307.26 µg QE/ml) and total phenolics (2856.33 µg GAE/ml). Fraction F3 contained 84.95% tannins, while fraction F13 contained 89.24% tannins.

Table 3 Qualitative phytochemical analysis of the *S. alata* fractions

Secondary metabolites	*S. alata* fraction	
	F3	F13
Alkaloids	+	-
Flavonoids	+	+
Phenolics	+	+
Tannins	+	+
Steroids	-	+

+: positive; -: negative

Table 4 Quantitative phytochemical analysis of the *S. alata* fractions

***S. alata* fraction**	**Flavonoids (µg QE/ml)[a]**	**Phenolics (µg GAE/ml)[b]**	**Tannins (% contribution)**
F3	4307.26	2856.33	84.95
F13	1614.96	592.36	89.24

a: equivalent to quercetin (QE); b: equivalent to gallic acid (GAE)

Examination of treated bacteria by SEM

The effects of *S. alata* fractions upon MRSA and *P. aeruginosa* morphology were investigated using scanning electron microscopy (SEM). Untreated MRSA and *P. aeruginosa* cells both showed normal cell morphology (Fig. 1A and Fig. 1B). In MRSA treated with fraction F3, the cells appeared shrunken (Fig. 1C), while in MRSA treated with fraction F13, the cells had a crystalline rough surface and appeared to be bursting (Fig. 1E). *P. aeruginosa* cells treated with fraction F3 (Fig. 1D) appeared dimpled compared to the untreated cells (Fig. 1B), whereas *P. aeruginosa* cells treated with fraction F13 had a slightly roughened surface and elongated cell shape (Fig. 1F).

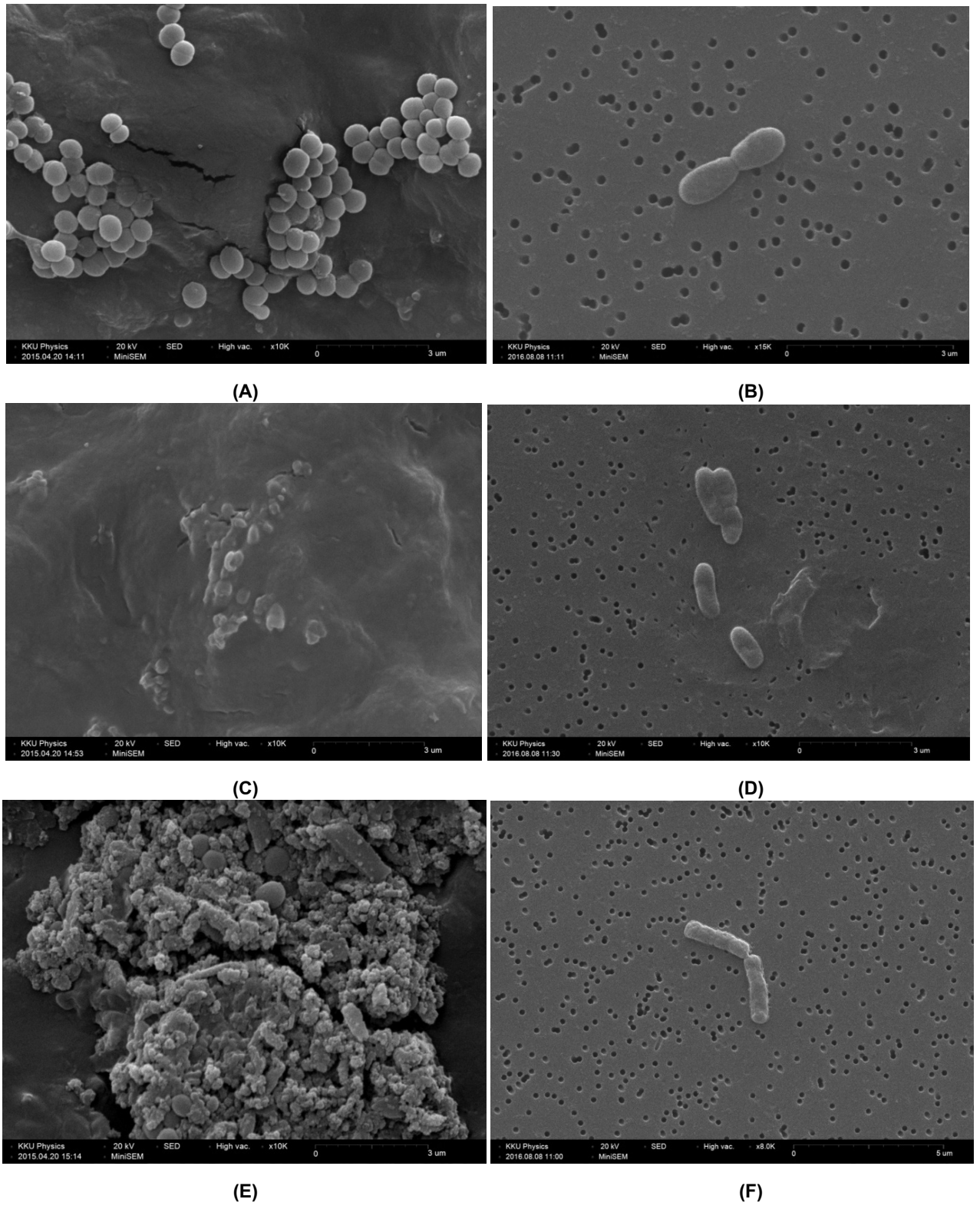

Figure 1 Scanning electron micrographs of (A) untreated MRSA cells, (B) untreated *P. aeruginosa* cells, (C) MRSA cells treated with *S. alata* fraction F3, (D) *P. aerugionsa* cells treated with *S. alata* fraction F3, (E) MRSA cells treated with *S. alata* fraction F13 and (F) *P. aeruginosa* cells treated with *S. alata* fraction F13.

Discussion and Conclusions

In this study, the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of 13 *S. alata* fractions were determined and compared with those of the crude extract. Of these fractions, F13 showed the most potent activity against MRSA and F3 showed the most potent activity against *P. aeruginosa*. The differences in the values of MICs/MBCs of each fraction suggested a different antibacterial activity of the fractions. Comparison of the antibacterial activities of the fractions with the crude extract by activity index indicates that several fractions are more potent than the crude extract. This result supports the fact that active compounds can be more concentrated in the fractions than the crude extract.[24-26] Another possibility might be explained by antagonistic effects between components within the crude extract that would reduce the antibacterial efficacy.[27]

Our results also show that Gram-positive MRSA is more sensitive to many of the fractions than Gram-negative *P. aeruginosa*, a finding which correlates well with previous medicinal plant studies.[25,28] This difference in sensitivity of the Gram-positive and Gram-negative bacteria to fractions might be due to differences in the cell structure of these microorganisms. Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is highly hydrophobic. This makes the cell wall of Gram-negative bacteria less permeable to antimicrobial substances present in plant extracts.[25,29-31]

The effects of the most active fractions (F3 and F13) on bacterial cells were also studied by SEM, and changes in bacterial cell morphology were observed. Our results are similar to previous studies in which secondary metabolites altered cell morphology and caused cell lysis.[6,32,33]

Subsequently, qualitative and quantitative phytochemical analyses of fractions F3 and F13 were performed. Qualitative analysis revealed the presence of flavonoids, phenolics, and tannins in both fractions, whereas alkaloids and steroids were only detected in fractions F3 and F13, respectively. Quantitative analysis showed that fraction F3 had a higher flavonoid and phenolic content but lower tannin content than fraction F13. In general, phytochemicals exert their antibacterial activity through different mechanisms. Alkaloids, for example, cause cell lysis and inhibit cell division.[32,34] Flavonoids block nucleic acid synthesis and disrupt the cytoplasmic membrane.[35] Steroids associate with membrane lipid and exert their action by causing membrane leakage.[36] Tannins decrease bacterial proliferation by blocking key metabolic enzymes.[37-39] Phenolics inhibit bacterial enzymes via oxidized compounds or through more non-specific interactions with proteins.[40,41] Although antibacterial activity has been detected from these classes of compound previously,[42] the efficacy of a medicinal plant may be due to the combined action of several different compounds rather than just one.[43,44] Therefore, the presence or absence of some compounds, variations in the quantities of these compounds, and variations in the mode of action of these compounds may be responsible for the variations in antibacterial activity and morphological changes seen with the different *S. alata* fractions.[29,45]

Our study shows that different *S. alata* fractions exhibit various levels of inhibitory and bactericidal activity against MRSA and *P. aeruginosa*. The effect of the fractions on bacterial cell morphology was fraction- and species-dependent as well. Further studies are now warranted to determine the synergistic effects of *S. alata* fractions and investigate their mode of action in *vivo*.

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