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# Effects of Butanol Extract of *Urtica dioica* on MRSA: Structural Degeneration Study

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**Abstract**
The aim of this study was to evaluate the antibacterial activity such as time-kill study of the butanol extract from *Urtica dioica* against methicillin-resistant *Staphylococcus aureus* (MRSA) using standard microbiological procedures. The effects of the butanol extract on MRSA cells was also studied by means of scanning electron microscope (SEM) and the results showed that the butanol extract caused internal shrinkage of the cells and completely collapsed the cells after prolonged exposure to it. Minimum Inhibitory Concentration (MIC) value was 16.67 mg/mL. The average log reduction in viable cell count in time-kill assay was $\geq$ 4.5 $\log_{10}$ CFU/mL using 33.33 mg/mL (2MIC) for 48 h.The extract was bactericidal against MRSA at 2MIC from 44 h interaction period. However, the time-killed assay suggested that the butanol extract of *U. dioica* significantly inhibited the cell growth and possessed bactericidal activity at concentration.
**Keywords:** *Urtica dioica*, butanol extract, MRSA, time-kill study, scanning electron microscope (SEM)

## INTRODUCTION

*Staphylococcus aureus* infections range from common skin infections, such as furunculosis and impetigo, to sever deep-seated infections *S. aureus* ranks first or second among bacterial pathogens which causing bloodstream infections. On the other hand, clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) have become the most common cause of infections among pathogenic bacteria around the world [1]. However, some diseases such as endocarditis, toxin shock syndrome, nosocomial infections and pneumonia are related to them [2] and also it is resistant to many available antibiotics [3].

Medicinal plants contain a rich source of antimicrobial agents. Hence, plants are used medicinally in different countries and are a source of many potent and powerful drugs [4]. Among *Urtica* species, *Urtica dioica* and *Urtica urens* have already been known and therefore consumed for a long time as medicinal plants in many parts of the world [5]. The antibacterial activities of extracts from *Urtica dioica* has been reported [6]. Furthermore, *U. dioica* has been consumed for a long time as medicinal plants in many part of the world [5] and were reported to treat stomachache in Turkish folk medicine [7] and also in Iran [8,9] as well as to treat rheumatic pain and for cough and colds [10]. In this concentration, we report the MIC and MBC values of the butanol extract against MRSA and also its effect on the cell degeneration.

## MATERIALS AND METHODS

**Preparation of the extract**

The aerial parts of *U. dioica* including leaves and stems were collected in Iran from Salmanshahr city in Mazandaran province (the geographical coordinates given by GPS: latitude: 36º42'34" N- 51º08'57" E and altitude: 21m) and Tehran city in Tehran province (the geographical coordinates given by GPS: latitude: 35º50'21" N- 51º25'22" E and altitude: 2012m) in August2007. The Voucher specimens were deposited at the Herbarium of the School of Pharmaceutical Sciences, University of Tehran (Iran) in April 2010 by the code of 6725-TEH.

Plant materials were washed under using tap water rinsed and dried under sunlight before ground to powder form. The dried powder form of plant material was kept at cool and dry place until further used.

The method of extraction included a five solvent systems (by using partition technique). A hundred gram of dried sample was extracted in 500 mL of 100% methanol solvent by using the Soxhlet extractor for 72 hrs at room temperature (30±2°C). Then the mixture was filtered using muslin cloth and followed by 150mm Whatman No. 1 filter paper. Approximately 300mL of the methanol extract was concentrated with a rotary evaporator (Heidolph). Concentrated methanol extract obtained was left in fume cupboard to dryness. The remaining methanol extract (liquid form) at volume 200mL was added with chloroform: distilled water (1:2) for further partitioning process. This mixture was carried out at volume 50mL for three times (total up was 250mL per times) in a separating funnel and shook slowly until two layers were formed. The bottom layer was collected as the residue (because density of chloroform is1.498 and had high gravity than other solvent in this mixture). Aqueous layer was formed at upper layer and was collected for further partitioned with diethyl ether. Aqueous layer was added with diethyl ether (50 mL in three times) as third solvent system for solvent- solvent partitioning. The upper layer was collected as diethyl ether fraction, while the aqueous layer at the bottom was collected as the residue. Subsequently, the aqueous residue formed was partitioned with ethyl acetate. The upper layer was collected as the ethyl acetate fraction while the bottom part was collected as the aqueous residue. The same partitioning process was repeated with butanol. All extracts were concentrated using the rotary evaporator and dried in the fume cupboard until they turned into paste form. Finally, the extracts obtained from this method were methanol extract, chloroform extract, diethyl ether extract, ethyl acetate extract and butanol extract as described by Mellidis and Papageorgiou (1993) [11]. The dried extracts were then weighed using microbalanceand were kept in 4°C. Regarding to best antimicrobial activity, butanol extract was selected for further study.

**Test isolate**

The MRSA culture which was previously isolated from a clinical sample taken in Hospital Seberang Jaya, Penang, Malaysia was provided by the Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang Island, Malaysia. The culture was grown on Nutrient Agar (NA) plates (Merck) at 37 ºC. The inoculum suspension was prepared by picking five single colonies from a 24-h-old culture and putting them into 5 mL of sterile physiological saline. The turbidity of the inoculum suspension was adjusted with sterile saline to match the turbidity of a 0.5 Mc-Farland standard.

**MIC and MBC determination**

The minimal inhibitory concentration (MIC) of the bacterial extract was determined by a broth micro-dilution assay in RPMI 1640 medium (Sigma) containing 0.2% dextrose buffered with 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS) to a pH of 7.0 at 25 ºC. The broth micro-dilution assay was conducted in a sterile, 96 wells, U-shaped, microtiter plate according to Jorgensen and Turnidge (2007). A two-fold dilution of the bacterial extract was prepared with sterile medium and 100 µL of the extract was dispensed into each well on the microtiter plate. On the same day, 100 µL of bacteria inoculum at approx$1.0\times10^5$ CFU/mL bacterial cells was added to each well, for a final volume of 200 µL. Amoxicillin was used as the reference drug, and a control with 5% DMSO and bacteria inoculum was included. After a 24 h-incubation at 37 ºC in a rotary shaker, 40 µL of 0.2 mg/mL p-iodonitrote trazolium violet salt (INT) (Sigma) dissolved in 99.5% ethanol was added to each well as a growth indicator. The color of INT changed from yellow to purple where microbial growth occurred. The MIC was recorded as the lowest concentration of extract that inhibited the growth of bacteria. The minimum bactericidal inhibitory (MBC) of the extract was also determined. After the MIC was read at 24 h, the viable cells were enumerated on SDA plates by a standard plates by a standard plate count. Inoculated plates were then incubated at 37 ºC for 24 h. The MBC was recorded as the lowest concentration of extract that resulted in 99.9% growth reduction relative to the control [12].

**Time-kill assay**

Butanol extract of *U. dioica* was prepared at a concentration of half of the MIC (½MIC), MIC and double time of MIC (2MIC) with NB broth against methicillin-resistant *Staphylococcus aureus*.

The 16 h bacterial cells grown on NA plates were suspended in 10 mL of sterile distilled water and the turbidity of the bacterial suspension was compared with 0.5 McFarland standard solutions ($1.0\times10^8$ cell/mL) and was adjusted to $1.0\times10^5$ cell/mL. Then, 1.0 mL of inocula and 1.0 of extract was added to aliquots of 10 ml NB in a 20 mL amber glass vial that give the final concentrations of ½MIC, MIC and 2MIC. The final concentrations of extract tested (final concentration at the retained ratio) for MRSA were 8.33 mg/mL (½MIC), 16.67 (MIC) and 33.33 (2MIC). An amber glass vial without the addition of extract (untreated, only inocula) served as control. Suspension of bacterial inoculum in the absence of crude extracts and nutrient broth agar including selected crude extracts incubated at 37ºC in convenient shaker (Certomat R) at a speed of 150 rpm for 48 hours. Finally, 0.5 mL portion was placed on NA plates and incubated at 37ºC for 24 hours. The growth of bacteria was counted and expressed as colony forming unit (CFU/mL). The growth of bacteria was measured (during the incubation period ranging from 0 hour to 48 hours) every 4 h for 48 h continuously by the above method. Also the test was done in triplicate [13,14]. The behaviour of the two pathogens was

followed by determining the initial and remaining viable counts at every 4 h of contact with the extract dilutions. All experiments were performed in triplicate. The graph curves were fitted using the software GraphPad Prism version 5.01 (GraphPad Software, Inc. 2007). The doubling time is the generation time of the bacteria. Generation time is following by formula by Todar (2012); (G) is expressed as the time (t) per generation (n = number of generations). Hence, G = t/n is the equation for calculations of generation time [15].

n (number of generations) = (log cells at end of incubation) – (log cells at beginning of incubation)/ 0.301

**Scanning Electron Microscope (SEM) observations**

SEM observations were used on the treated bacterial cells. One mL of the bacterial cell suspension at the concentration of $1\times10^5$ cells/mL was inoculated on Nutrient agar plates and were incubated at 37 ºC for 24 h. Two milliliter of the butanol extract (at a concentration of MICs, 16.66 mg/mL for MRSA) was then pipetted on to the inoculated agar and was further incubated for another 12, 24 and 36 h at the same incubation temperature. An inoculated Nutrient agar plate without the addition of butanol extract was used as control cells (0 h). A small block of bacteria (1.0 $cm^3$) containing agar was cut and withdrawn from the inoculated plate at 0, 12, 24 and 36 h, and was fixed for SEM observation. The prepared samples were observed under the SEM (Fesem Leo Supra 50 VP, Carl Zeiss, Germany).

## RESULTS

**Time-kill study**

The time-kill study was performed over a period of 48 h with bacteria being exposed to ½MIC, MIC and 2MIC values. In this research, the growths of MRSA was evaluated.

The growth of MRSA cells without the addition of the extract showed a normal growth curve pattern with lag, log (exponential), stationary and death phases (Figure 1). After the addition of the butanol extract at ½MIC value (8.33 mg/mL), the growth pattern of the MRSA cells were almost the same as the MIC (16.67 mg/mL) but with lesser colony forming unit per millilitre (CFU/mL). However, the maximum number of CFU at the end of exponential phase (32 hours of cultivation time) for control was $1.14\times10^4$ CFU/mL while for ½MIC (half MIC); the number did not achieve. This conditions could be due to the concentration of ½MIC (8.33 mg/mL) and MIC (16.67 mg/mL), where the MRSA cells growths were inhibited significantly by the extract. The results also revealed that there was no distinct exponential phase seen. At the concentration of 2MIC (33.33 mg/mL), the growth curve exhibited almost a stagnant line with no significant growth of the cells throughout the incubation period.

Referring to Table 1, the MBC of MRSA exposed to butanol extract was 33.33 mg/mL but regarding to Estimated Reduction (ER), results showed 8.33 mg/mL after 24 h and 16.67 mg/mL after 48 h. This result might lead to the conclusion that a reduction of 4 $\log_{10}$ cycles was reached at one level lower than the results obtained before. However, both of the results exhibited the same MBC values with slight differences.

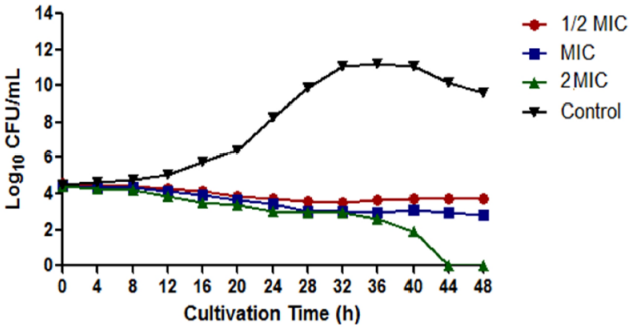


**Figure 1.** Representative time-kill curve plot of butanol extract of *Urtica dioica* against MRSA at the following concentrations: control (without extract); 8.33 mg/mL (½ MIC); 16.67 mg/mL (MIC) and 33.33 mg/mL (2MIC).

**Table 1.** Mean populations ($\log_{10}$ CFU/mL) and estimated reductions of MRSA against butanol extract respectively, after exposed to different concentrations of extracts for 24/48 h

| | Butanol extract against MRSA | | | |
|---|---|---|---|---|
| | Mean ± SEM | | ER | |
| | 24 h | 48 h | 24 h | 48 h |
| **Control** | 8.281±0.11 | 9.604±0.02 | NA | NA |
| **2MIC** | 3.000±0.01 | 0 | 5.2 | 0 |
| **MIC** | 3.452±0.06 | 2.824±0.07 | 4.8 | 6.8 |
| **½ MIC** | 3.692±0.02 | 3.692±0.16 | 4.6* | 5.9 |

NA = Not applicable
Control = Initial inoculum
ER = Estimated reduction = (Log10 CFU/mL of control at 24/48 h) – (Log10 CFU/mL after 24/48 h of exposure to the treatment
* MBC since > 4 log10 CFU/mL reduction was achieved.

The generation time for MRSA at control (without the addition of extract) was 59.18 min (from 20 to 32 h). However, when butanol extract of *U. dioica* was tested, the greatest reduction in MRSA was $\geq$ 4.5 $\log_{10}$ CFU/mL using 33.33 mg/mL (2MIC) for 48 h. Furthermore, MRSA viability was completely eliminated after 44 hours.

**Effect of butanol extract of *Urtica dioica* on the cell of MRSA**

The present investigation on the effect of the butanol extract of *U. dioica* on the exterior parts of the MRSA cells has brought conformity to the screening part of the antimicrobial activity noted earlier. Hence, some interesting observations were obtained from SEM micrographs.

Figure 2 represents the effect of extract at the concentration of MIC value on the MRSA cells. SEM analyses were performed and demonstrated the altered cell morphology as compared to control group. The untreated cells of MRSA in Figure 2 A shows the normal morphology with a regular, smooth surface and the cells retained their coccal morphology and seemed to be normal. Figure 2 B

shows the 12 hour of exposure to the extract where the cells undergone some changes in their cell morphology. The cell surface showed irregularities and the individual cells undergoing to shrunken phenomenon. There were cavities formed and this condition seems to be resulted from the leakage of the cell cytoplasm. At a closer view, the coccal shaped cells became like donut shaped cells. Figure 2 C exhibits the 24 hours of butanol extract treated cells which revealed severe detrimental effect on the morphology of the cell membrane. The surface of the cells were crumpled and shrunk. This condition was then followed by the subsequent formation of cavities or pores on the surface of the cells (arrow sign) after 24 hours of exposure. It may relate to the loss of cellular materials from the cells and thus caused some of the cells lost their coccal-shaped and finally exhibited indistinctive shapes as compared to the control cells. Figure 2 D shows the 36 hours of butanol extract treated cells which the cells exhibited physical damages (ruptured walls and irregular shape of cells) showed. This obviously suggests that some cells undergone lysis and became completely disrupted. The cells were totally damaged and collapsed to the extent of losing their original coccal-shaped, and wrinkled abnormalities were seen with numerous small clefts (arrow sign) formed. It is also noticed that the majority of the treated cells were distinguishable from untreated cells.

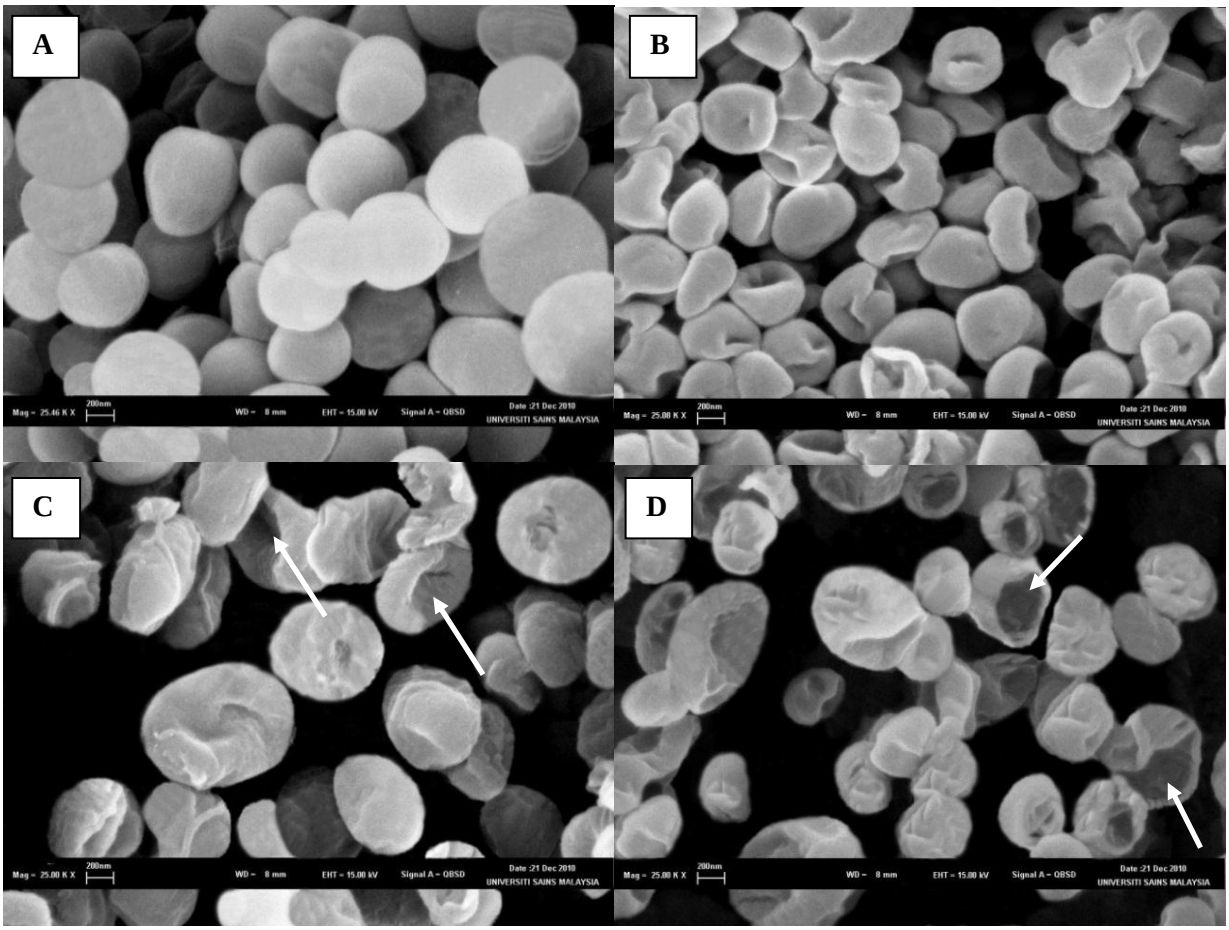


**Figure 2.** SEM micrographs of the effect of butanol extract on MRSA at different time of exposure. Control bacterial cells without treatment (25000×) **(A)**; MRSA treated for 12 hours (25000×) **(B)**; MRSA treated for 24 hours (25000×), the red arrows indicated the formation of cavities or pores on the bacterial cells **(C)** and MRSA treated for 36 hours (25000×), the red arrow indicated the formation of pores on the bacterial cells **(D)**.

## DISCUSSION

All levels of extracts in time-kill assay exhibited the inhibitory effect by reducing the number of MRSA cells by butanol extract in relative to the controls. Significant reductions were obtained at the concentration of 2MIC but the maximum reductions achieved were almost 4 $\log_{10}$ (> 4 $\log_{10}$), indicating that at this concentration the extract had the bactericidal effect. Bactericidal activity was defined as a ≥ 3 $\log_{10}$-fold decrease in the number of survivors at each time point compared with the number inoculated at time zero. This activity was equivalent to 99.9% killing of the inoculums [16]. This MRSA strain only demonstrated the concentration-dependent effect, but not the time dependent effect.

MBC is defined as the lowest concentration of antimicrobial agent that kills ≥ 99.99% of the test inoculum. In another words, MBC is the lowest concentration that yields a 4 log CFU/mL reduction from the initial level of inoculum [17]. Also, there is accumulating evidence that sub-MICs of antimicrobial agents which unable to kill bacteria, can modify their physico-chemical characteristics and the architecture of their outermost surface and may interfere with some of the bacterial functions [18].

The antimicrobial activity of *U. dioica* is due to the presence of an array of phytochemicals, but most importantly due to the activity of a flavone named 5-hydroxytryptamine (5-HT) [19,20]. The flavone may act directly on microorganisms and result in growth inhibition by disrupting cell membrane synthesis or synthesis of essential enzymes. Cushnie and Lamb (2005) reported that the antibacterial activity of flavonoids is related to damage of the bacterial membranes, causing an increase in the permeability of the inner bacterial membrane, and dissipation of the membrane potential [21]. Different groups of researchers have reported a common structural feature which is related to hydroxylation of the B-ring. The mechanism of action of the flavan-3-ol (catechin, epicatechin: EC, epigallocatechin: EG and epigallocatechin gallate: EGG) is related to the damage of the bacterial membranes. The low catechin susceptibility of Gram-negative bacteria may be partially attributable to the presence of lipopolysaccharide acting as a barrier. In the case of the flavonols (quercetin, fitesin, rutin and hesperidin) it was shown that they caused an increase of the permeability of the inner bacterial membrane and dissipation of the membrane potential [22,23]. Also the presence of phenolic compounds in the butanol extract exhibiting that may be the responsible for antagonistic property of this plant [24].

This assay correlates with the pharmacodynamics and pharmacokinetic effects of the *in vivo* conditions. The butanol extract was manipulated at several MIC levels (MIC, half MIC and double MIC) which would predict the efficacy of the extract *in vivo*. However, the time-kill assay more closely simulates to determine the interaction between antimicrobial agents and strain of bacteria [25].

In addition, the generation times of the exponential phase for the growth profiles were determined. By definition, generation time is the period of time required for a bacterium to perform one round of binary fission process to produce two identical bacterial cells [14]. However, the rate of exponential growth of a bacterial culture is defined as generation time, also the doubling time of the population of bacteria in culture.

On the other hand, lectins of different carbohydrate specificities are able to exert growth inhibition or death of fungi and bacteria. Antibacterial activity on Gram-positive and Gram-negative bacteria occurs through the interaction of lectin with components of the bacterial cell wall including teicoic and teicuronic acids, lipopolysaccharides and peptidoglycans. *U. dioica* containing lectin. They are toxic as they can impair tissue function but our human body can replace any damaged cells faster than they are destroyed [26,27].

Continued further exploration of plant-derived antimicrobials is needed today. Further study is necessary to determine the identity of the antibacterial compounds from this plant and also to determine their full spectrum of efficacy. However, this study of *in vitro* antibacterial evaluation of butanol extract of *U. dioica* forms a primary platform for further phytochemical and pharmacological studies to discover new antibiotic drugs.

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