Antibacterial Activity of Allium ampeloprasum Against ESBL\(^{+}\)ve *Escherichia Coli* and Methicillin-Resistant *Staphylococcus Aureus* by Transmission Electron Microscopy

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**KEY WORDS**
Allium Ampeloprasum; MRSA; ESBL; Transmission Electron Micrograph; Herbal Medicine

**ABSTRACT:** Infectious diseases caused by MDR pathogens are one of the primary health concerns globally, affecting millions of people every year. Infectious diseases are responsible for high morbidity throughout the world, accounting for about a quarter of deaths around the globe. This growing problem has necessitated the exploration and development of novel antimicrobials such as natural products, plant phytochemicals and fabricated antibiotics with less or no side effects. Medicinal plants are known to be valuable resources for the development of novel antimicrobial drugs. Medicinal herbs are better equipped at metabolic Bio-engineering of active compounds than conventional laboratory approaches. Preliminary antibacterial assay by agar well assay of Allium Ampeloprasum on MDR pathogens MRSA and ESBL\(^{+}\)ve *E.coli* have shown significant antibacterial activity. After clear zone of inhibition formation, the further evaluation of antimicrobial activity was performed by Transmission Electron Microscopy (TEM), and morphological changes are observed at the cellular level. Ultrastructure observations have demonstrated severe damage to both the bacterial cells of MRSA and ESBL\(^{+}\)ve *E.coli*. For the first time, the antibacterial activity of Allium leaf extract against MRSA and ESBL\(^{+}\)ve *E.coli* was confirmed by using TEM micrographs.

**INTRODUCTION**

The emergence of multidrug-resistant (MDR) microbes, aroused by overuse of antibiotic drugs has led to a marked decrease in the efficacy of commonly used antibiotics, which has now become a severe problem worldwide (Cragg and Newmann 2013; Ventola 2015). There has been an upsurge in the number of reports about the resistance development towards various antimicrobials, which are available in the market currently. There has been a tremendous rise in the incidences of multi-drug resistant pathogens with complex mechanisms to escape the antibiotic action (Davies and Davies, 2010). During the past 30 years, 37 new human pathogens have been reported to be as disease-causing agents in human. Medicinal plants are recognized as treasured resources for novel antimicrobial drugs development (Cowman, 1999). Medicinal herbs are better fortified at metabolic Bioengineering of active compounds when compared to conventional laboratory approaches (Pan et al, 2013). Several studies are focusing currently on many natural medicinal herbs, which are often used in folkloric medicine and known to have a high probability of emerging as a new source for bactericidal or bacteriostatic agents (Verma and Singh, 2008).

Development of novel antimicrobial phytochemical compounds is one of the strategies employed by unleashing the hidden potential of natural resources such as medicinal plants (Dias 2012). There are several advantages in using herbs or natural products as an alternative source for the development of novel antibacterial compounds. Medicinal plants are known to produce diverse complex compounds, which are still beyond the capability of synthetic chemists.
Moreover, there is a minimal risk of side effects associated with medicinal plants as compared to synthetic drugs, as the edible plants or traditional medicine are reported to have decidedly less or no side effects, along with the availability and cost-effectiveness (Ekor, 2014).

MRSA and ESBL +ve E.coli are major MDR pathogens that are responsible for causing life-threatening human diseases (Santajit and Indrawattana, 2016). The emergence of new resistant strains of MRSA and ESBL +ve E.coli have uncovered virulent growth and enhanced ability to cause disease in a healthy individual (Joainig 2010). The use of antibacterial drugs has been increasing to kill the bacterial pathogens and safeguard the public health. A series of antimicrobial substances, including quaternary ammonium compounds, antibiotics, metal ions, can target and inhibit bacterial growth and in turn, damage the morphological features of microorganisms (Rudramurthy et al., 2016). An essential objective in the discovery of antimicrobial drug is the novel compound development with the potential of killing resistant pathogens, by various novel modes of action, which could hinder the advent of resistance in the microorganisms (Nicolau and Rigol 2017). Medicinal plants are considered as asignificant source for new antimicrobial drugs discovery. Allium ampeloprasum is one of the plants, which has shown tremendous therapeutic potential including anti-microbial activity. The essential oil of A. ampeloprasum comprises 22 compounds, which are the main constituents of the plant and were identified by examining the aerial part of the plant by GC and GC MS. Allium ampeloprasum contains the antimicrobial agent allicin, which contains diallyl sulphide, and thiosulphinate, which targets pathogens affecting the food (Huang and Ren 2013).

In the present study, we have used leaf extract of Allium ampeloprasum to assess the antibacterial activity against MDR pathogenic bacterium. We have used hexane treated ethanolic extract from the leaves of Allium Ampeloprasum. MRSA and ESBL +ve E.coli were used as model bacterial species to study and evaluate its antibacterial activity by using Agar well assay followed by Transmission Electron Microscopy (TEM). Among the various approaches used for studying the antimicrobial activity of the plant, TEM is one of the imaging-based techniques used in the detection of structural alterations in the cell morphology (Jong et al., 2011).

MATERIALS AND METHODS

PLANT COLLECTION AND PROCESSING

Fresh leaves of Allium ampeloprasum were collected from the local vegetable market in Jeddah, Saudi Arabia. Plant leaves were sluiced under running tap water, dried under the shade on filter papers and then grounded into a fine powder using an electric blender. The extraction of Allium was carried out by using ethanol as a solvent. Dried plant materials were sequentially extracted by mixing 20g of leaves with 100ml of hexane followed by 100 ml of ethanol in a shaking incubator for 48 hours at 25°C and 120 rpm. The mixture was then filtered using filter paper (Whatman No. 1). The solvent was dried entirely from the filtrate at reduced pressure and 40°C temperature in Rotavapor (BUCHI, Switzerland). The dried powdered leaf extract was stored at ~20°C until further use (Alamri et al., 2012).

BACTERIAL STRAINS

Clinical isolates used in the present study(Table 1) were obtained from the clinical microbiology laboratory of the King Abdulaziz University Hospital, Jeddah, Saudi Arabia. E. coli, ESBL +VE, isolated from deep wound sample, is resistant to trimethoprim/sulphamethoxazole and ciprofloxacin. Methicillin-resistant Staphylococcus aureus(MRSA), isolated from superficial wound sample, is resistant to clindamycin, erythromycin, oxacillin and cefazolin.

ANTI-MICROBIAL ACTIVITY

Anti-microbial activity of Allium ampeloprasum leaf extract was evaluated against two clinical pathogens Escherichia coli (Extended substrate β-lactamases +ve) and Methicillin-Resistant Staphylococcus Aureus (MRSA). Agar well diffusion assay method was employed for the determination of antibacterial activity of A. ampeloprasum.

Mueller Hinton agar plates were prepared, and 0.5 MacFarland units of bacteria were spread onto the agar plates and allowed to dry for few minutes. 6mm diameter of wells dug on agar plates for applying extract into it. 40mg/ml of Allium extract was poured into the wells and incubated at room temperature for one hour, followed by incubation at 300C for 24 hours. DMSO was used as a negative control and penicillin/streptomycin was used as a positive control.

TRANSMISSION ELECTRON MICROSCOPY

Samples of clinical isolates of E. coli and MRSA were examined using Transmission Electron Microscopy (TEM), as described by Haschemeyer and Meyers (Haschemeyer and Meyers 1972). Bacterial culture of both control and treated samples were transferred to a 300 mesh carbon-coated copper grid by keeping the grid coated-side-down on a drop of MRSA and ESBL E. coli
culture for about 2 min. Excess liquid was drained off by touching the grid with a piece of Whatman filter paper, and bacteria were then negatively stained by floating the grid on a drop of 2% (w/v) PTA solution (pH 6.5) for about 1 min. Excess liquid was again removed by touching the grid with a piece of Whatman No. 1 filter paper, and the final bacterial preparations of both control and treated bacteria were examined using TEM using a JEM-1011 microscope (Jeol, Tokyo, Japan) at 80 KV accelerated voltage (Ohiet al., 2014).

Table 1. Evaluation of antibacterial activity of leaf extract of Allium Ampeloprasum by Agar well assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Resistance phenotype</th>
<th>Diameter of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli ESBL +VE</td>
<td>Resistant to Trimethoprim/ Sulphamethoxazole and ciprofloxacin</td>
<td>15mm</td>
</tr>
<tr>
<td>S.aureus MRSA</td>
<td>Resistant to clindamycin, erythromycin, oxacillin and cefazolin</td>
<td>20mm</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>-</td>
<td>22mm</td>
</tr>
</tbody>
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RESULTS AND DISCUSSION

The antimicrobial activity of A. ampeloprasum leaf extract was tested against MDR Gram-positive and Gram-negative bacteria initially by agar well diffusion method. Leaf extract at 5mg/ml dose showed significant antimicrobial activity against MRSA strain and had shown the zone of inhibition of 20 mm in size (Table 1). Allium extract also exhibited antimicrobial activity against E.coli ESBL +VE strain with the zone of inhibition of 15mm in size (Table 1). In treatment with only DMSO solvent control, no zone of inhibition was detected.

Effect of Allium leaf extract on the morphology of pathogens was also investigated by negative contrast Transmission electron microscopy. After the exposure of MRSA and E. coli ESBL+ve cells with Allium leaf extract caused morphological changes including cell membrane damage and release of the cellular content. As observed under the microscope, many protuberances at outer membrane along with lysed membranes were seen. The TEM images revealed the formation of blebs on the bacterial surface. There was a clear indication of cell membrane damage, cellular disintegration, and release of cytoplasmic content, leading to cell death (Fig.1 and Fig.2).

The antimicrobial activity of leaf extract against MRSA cells was confirmed by observation of Electron microscopic images obtained after overnight incubation of MRSA cells with Allium leaf extract. The cells after treatment were observed with detectable shrinkage with a characteristic shape and irregular shape. MRSA cells have demonstrated aberrant morphology followed by cracking, rupturing and release of cytoplasmic content (Fig.1).

Morphological disparities in the bacterial cell wall organization may affect the efficacy of the Allium extract. Presence of thin peptidoglycan layer (2-3nm) between the cytoplasmic membrane and the outer membrane in Gram-negative bacteria when compared to Gram-
positive bacteria which lacks the outer membrane but contains a peptidoglycan layer of around 30 nm thickness is influencing the antimicrobial activity of Allium extract. This thicker wall is responsible for acting as a barrier protecting the cell from the entry of phytochemicals into the cytoplasm. The bacterial strain may also regulate the bioavailability of the plant phytochemicals present in the Allium leaf extract by inactivation or modification.

As observed in TEM micrographs of ESBL+ve E.coli, Allium leaf extract promotes detachment of the outer cell membrane and spilling off the cytoplasmic content (Fig.2). Moreover, the cell wall is severely damaged and showed undulations indicating a loss of integrity and the aggregated cytosolic material is seen on the cell surface. The cytoplasmic membrane of E.coli cells was separated and irregular from the outer cell membrane after the overnight treatment and release of the intracellular contents was observed. Treatment of MRSA cells with Allium leaf extract resulted in lysis of outer cell membrane with the formation of protrusions as noted in the TEM images. Moreover, the cellular contents were entirely leaked from the cytoplasm.

Cell membrane destruction of the Gram-negative E.coli ESBL +ve as observed in TEM analysis may be one of the possible modes of action for the phytochemicals present in Allium leaf extract. The release of intracellular material occurred due to damage to the membrane eventually leading to the cell death (Li et al., 2015). It has been claimed that the use and application of antibacterial agents that are liable for the disruption of membrane bi-layer is one of the most efficient approaches for inhibiting the bacterial growth. As these bioactive compounds are more valuable in being active against dormant bacteria, they are of great interest to study. Such organisms which are metabolically inactive will be able to survive at high doses of various other classical antibiotics, and long-term treatment is therefore required for drug efficacy (Hurdle et al., 2011); (Agnihotri et al., 2014)

**CONCLUSIONS**

Transmission electron microscopy has confirmed the antimicrobial activity of Allium leaf extract against MDR bacterial pathogens after observing the structural changes due to the action of phytochemicals present in the leaf extract. Electron micrographs of MRSA and ESBL +ve E.coli displayed profound alterations in the morphology of bacterial cells after exposure to Allium leaf extract. In both the pathogens, the cell wall disruptions resulted in the release of intracellular material and, ultimately leading to flaccid and empty cells. The cellular damage observed on TEM examination has shown the lethal effects of Allium leaf extracts on the pathogens. For the first time, the antibacterial activity of Allium leaf extract against MRSA and ESBL +ve E.coli was confirmed by using TEM micrographs. By isolating and purifying the phytochemicals responsible for antimicrobial activity and investigating its mechanism of action by using molecular studies can lead to the discovery of novel antimicrobial compounds from Allium ampeloprasum.

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