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Original research article

Antivirulence and wound healing effects of royal jelly and garlic extract for the control of MRSA skin infections

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ABSTRACT

Methicillin Resistant *Staphylococcus aureus* (MRSA) has become increasingly a major problem and responsible for most of the hospital acquired infections in Egypt. Thus, there is an urgent need for agents with new therapeutic targets particularly those targeting bacterial virulence. MRSA isolates were investigated for biofilm formation, adhesion and invasion assays. Thereafter, the effect of garlic extract and royal jelly on those virulence factors was tested. These two agents were tested *in vitro* and *in vivo* using an animal model of MRSA skin infection. The garlic extract caused significant reduction in biofilm formation (60% median reduction) however, it had no significant effect on bacterial adhesion. Royal jelly reduced biofilm formation and bacterial adhesion significantly (≥50% median reduction). Animal study revealed that royal jelly could eradicate MRSA completely and promote wound healing even in the uninfected groups. However, the wound in case of garlic treated groups was dry and no exudates or pus was observed indicating its antiseptic effect.

Conclusion: The results implied the significant role of royal jelly not only in eradication of MRSA infection, but also in promoting the healing process in the wounds.

1. Introduction

MRSA has been associated with nosocomial infections and rapidly developed resistance to multiple drug classes. It is now regarded as a major hospital acquired pathogen worldwide [1]. Methicillin resistance in *S. aureus* is due to the *mec*A gene which encodes an altered penicillin-binding protein (PBP2a) with decreased affinity to β-lactams [2,3]. Virulence is a multifactorial process and requires the use of a variety of components, many of which are coordinately regulated to allow the organisms to adapt to the host environment and become successful pathogens. These virulence determinants promote tissue colonization, tissue damage, and distant diseases [4]. The strategy of many antivirulence drugs is to target specific virulence factors that are unique in the pathogens pathogenesis thus inhibit specific mechanisms that promote infection and are essential to persistence in the pathogenesis cascade, and/or cause disease symptoms [5]. This approach lacks the disadvantages of the traditional treatment such as; side effects of chemotherapeutic agents; limited number of newly discovered antibiotics; and increasingly emerging resistance to existing antibiotics.

MRSA biofilm is an important virulence mechanism that complicates infections [6] as well as bacterial adhesion to tissues was a prerequisite step in the infectious process [7,8]. Thus, targeting bacterial biofilm and adherence of clinically relevant MRSA isolates using some natural products such as royal jelly and garlic extract was aimed. The *in vivo* testing of the therapeutically promising agents in an animal model system is an important stage in drug discovery as an antimicrobial drug and for establishment of their effectiveness [9–11]. An *in vivo* model utilizing animal skin infection has the advantage of simulating wound repair that is most similar to clinical cases. Therefore, the aim of our study was testing the effect of garlic extract and royal jelly on MRSA biofilm and adherence followed by *in vivo* testing of such agents using an animal model of MRSA skin infection.

2. Materials and methods

2.1. Bacterial isolates

A total of 16 MRSA isolates were previously recovered from different clinical specimens (collected from Ain Shams University

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hospital patients) and identified using cefoxitin disc diffusion method [12].

2.2. Animals

The study was conducted according to ethical guidelines (Ain Shams University, Egypt). Eight to nine weeks-old (about 110–120 g) female rat (Albino rats of Wistar strain) were used in this study. Rats were housed one per cage and fed with rat routine diet and water that are normally used in laboratory animal houses. Standard diet pellets (El-Nasr, Abu Zaabal, Egypt) contained not less than 20% protein, 5% fiber, 3.5% fat, 6.5% ash and a vitamin mixture.

2.3. Chemicals and culture media

Different chemicals used in the present study were of highest quality available and obtained from Sigma-Aldrich (Munich, Germany), El-Nasr chemical Co. (Adwic, Cairo, Egypt) and other local suppliers.

2.4. Garlic extract

Peeled fresh garlic (100 g) was chopped and homogenized in 100 ml sterile distilled water in a blender. After filtration through Whatman filter paper, the filtrate was further sterilized by passing through a sterile 0.22 μm-pore-size filter unit. The filtrate was collected in a sterile 50 ml falcon tubes and stored at 4 °C until used.

2.5. Royal jelly

Egyptian royal jelly packs were obtained from Imtenan Co. (Cairo, Egypt) and were stored at −20 °C until used.

2.6. Testing the effect of garlic extract and royal jelly against MRSA virulence determinants in vitro

2.6.1. Biofilm assay

The biofilm assay was carried out using spectrophotometric microtitre plate assay as previously mentioned [12–14]. The tested agent was prepared and incorporated to the require concentrations as follows: Fifty microliter of pre-determined concentration(s) of the test agent was incorporated in the bacterial cell suspension (150 μl) during biofilm assay. Control was treated similarly except that 50 μl saline were added instead of the test agent. Biofilm persistence in the presence of the test agent was calculated according to Tré-Hardy et al. [15] using the following formula:

$$\%\ \text{change in biofilm} = \frac{[A_{50}(\text{control}) - A_{59}(\text{agent})]}{A_{59}(\text{control})} \times 100$$

where A_{590} means absorbance at 590 nm [15].

2.6.2. Adherence and invasion assays

This was carried out as described by Plotkowski et al. [16] with some minor modifications: After removing the media and washing the monolayer twice with PBS, 100 μl aliquots of the bacterial suspension (adjusted to 10^8 CFU/ml) were added to the wells (8 wells for each isolate and one well acted as a control; contained PBS free cells). Following 2 h of incubation at 37 °C, the non-adhered bacterial cells were removed by decantation and the wells were washed 3 times with PBS. Lysis of *Vero* cells was carried out by adding 125 μl of mammalian cell lysis solution in each well and incubated at 37 °C for 30 min. The cell lysates were then collected from the 8 wells appropriately diluted in saline and 100 μl of each dilution was uniformly plated in duplicate on nutrient agar. The number of viable bacteria was determined by counting the CFU for each plate and multiplying the average count by the dilution factor to give the total number of associated bacteria (adherent and invaded). Gentamicin survival assay was used to determine number of internalized bacteria [16].

2.7. In vivo *testing of royal jelly and garlic extract as anti-virulence agents in animal model of MRSA skin infection*

2.7.1. Testing microbial virulence in animal model with MRSA skin infection

This test was carried out to ensure that the selected MRSA isolate (MR_{16}) is pathogenic and can cause soft tissue skin infection to rat model with skin injury as well as to study the time course of infection. Two groups were included (five rats each): one consisted of injured uninfected untreated rats (control; group I) and the other consisted of injured infected untreated rats (test; group II). The test was carried out as described below.

2.7.2. Testing the effect of royal jelly and garlic extract in animal model of MRSA skin infection

Establishment of a superficial skin infection model in rats was carried out using “Tape or sharp tool stripping infection model” according to Dai et al. [17], Aguiar et al. [18] and Tsao et al. [19] with some minor modifications as follows: Overnight culture of a selected MRSA isolate (MR_{16}) in 5 ml TSB broth was diluted with PBS and adjusted to contain 10^8 CFU/ml. For infection, adjusted MRSA culture was applied to the injured skin in a volume of 200 μl. Tape or sharp tool stripping infection model was carried as follows: The rats were anaesthetized by exposure to 2% diethyl ether presented on a cotton ball or gauze pad by the “open-drop” technique for 5–10 min (diethyl ether was approved for animal anaesthesia in the protocol reviewed by the Animal Care and Use Committee “ACUC”). The fur of the front back was stripped from the rat using hair removal machine. An area of 2 cm^2 at the one third of the back of the rat was injured using tape or sharp tool. Following the back injury, the skin became visibly damaged and was characterized by reddening and glistening but no regular bleeding. Macroscopically, this procedure resulted in the controlled removal of most of the epidermal layer, with only a few basal epidermal cells remaining. After skin injury, the bacterial infection was initiated by placing 200 μl droplet of adjusted bacterial suspension (adjusted to a final optical density corresponding to 10^8 CFU/ml) on the injured area. The first application of either tested agents to the injured skin of the rats was at 4 h post-infection. Thereafter, at 16 h after the first treatment, additional application of the tested agents was made and this was repeated twice daily (in the morning and the evening, with an 8 h interval) for a period of 10 days. For each treatment 1 mg of the tested agent was applied per gram of the rat body weight (dose: 1 mg/g/twice daily) [17–19].

Four control and four test groups, five rats each, were included in the experiment. Monitoring of the results was carried out by measuring the wound diameter (3 different readings for each diameter and taking the average), photographing the wound site by digital camera each other day and by histopathological examination of biopsy specimens of representative rats taken from each group. These include:

Group I (GPI): control, intact, non-infected, untreated
Group II (GPII): control, injured, infected, vehicle
Group III (GPIII): control, injured, non-infected, untreated
Group IV (GPIV): control, injured, infected, untreated
Group V (GPV): test, injured, infected, 50% royal jelly (R50)
Group VI (GPVI): test, injured, infected, 100% royal jelly (R100)