International Journal of Phytomedicine 4 (2012) 150-154
http://www.arjournals.org/index.php/ijpm/index

Short Communication

ISSN: 0975-0185

Leucasin - Induced Cytoplasmic Membrane Damage in *Staphylococcus aureus*

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Abstract

Leucasin is one of the active antimicrobial principle of *Leucas aspera*. The effect of this compound and other antibacterial agents with known mechanisms of action upon the cytoplasmic membrane integrity of *Staphylococcus aureus* was investigated by comparing scanning electron microscopy (SEM) and potassium loss profiles from bacterial cell suspensions. The minimum inhibitory concentrations (MICs) of leucasin, novobiocin - the bacteriostatic antibiotic and penicillin G – the bactericidal antibiotic against *S. aureus* (ATCC 12600) were determined as 35 µg/ml, 55 ng/ml and 40 ng/ml respectively. The morphology of *S. aureus* was impaired, when treated with leucasin showing mucilaginous mass, which could lead to the impairment in cell division, as observed under SEM. When *S. aureus* were suspended in potassium free media containing 35 µg/ml leucasin, a 100 fold decrease in viability was observed after 12 h. Potassium loss assay revealed that *S. aureus* treated with 35 µg/ml leucasin lost 17% more potassium than untreated control populations whereas, cells treated with 40 ng/ml of penicillin G exhibited 9% increase in potassium loss and 55 ng/ml of novobiocin had no effect on potassium loss. This data may be attributed to either direct damage to the cytoplasmic membrane or indirect damage affected through autolysis/weakening of the cell wall and consequent osmotic lysis.

Keywords: Leucasin; Mechanism of action; *Staphylococcus aureus*; Cytoplasmic membrane damage; Potassium loss.

Introduction

Flavonoids are natural plant pigments, reveal large spectrum of biological activities [1] and have been isolated from fruit, vegetables, nuts, seeds, stems, flowers and leaves as well as tea, wine. For centuries, preparations of flavonoids have physiologically active constituents used by physicians and lay healers to treat human diseases [2]. Leucasin (5,7-dihydroxy-2-[14-methoxy-15-propyl phenyl]-4H-chromen-4-one) a novel flavone, associated with such preparations [3]. Leucasin is one of the major constituent of *Leucas aspera*, a perennial herb used by South Asian indigenes to treat infections. It has been found that *L. aspera* was shown to have anti inflammatory [4], larvicidal and antinociceptive effects [5]. Recently, from our own laboratory leucasin was characterized and shown to have antioxidant property [3].

If mechanism of action could be established, this would assist in the optimization process and provide a focus for toxicological attention and aid to tackle the future resistance problems and may ultimately lead to the development of new antibacterial compounds [6]. It has been suggested that certain flavonoids including (-) epigallocatechin gallate [7], sophoraflavanone G [8] and quercetin [9] cause damage to the cytoplasmic membrane of bacteria and is responsible for antibacterial effect. The current study, took into consideration that the mechanism of action of leucasin anti – *Staphylococcal* activity involves damage to the *S. aureus* cell membrane. Generally, the first sign of an increase in cytoplasmic membrane permeability is provided by morphological changes in cells and also leakage of the important intracellular solute, potassium. In recent years, measurement of potassium loss from bacterial cells has successfully been used to detect membrane damage caused by phytochemicals [10]. Therefore, leucasin treated cells of *S. aureus* were examined for morphological changes under scanning electron microscope and cytoplasmic membrane damage by measuring loss of internal potassium.

Materials and methods

Chemicals

BSA (Bovine serum albumin fraction V), dimethyl sulfoxide (DMSO), Disodium hydrogen phosphate (anhydrous), Sodium dihydrogen phosphate, Novobiocin (sodium salt) and Penicillin G, Sodium chloride, Nutrient agar were obtained from HiMedia Pvt Ltd., Mumbai, India.

Characterization of leucasin

From our own laboratory, leucasin was isolated and characterized from the leaves of *Leucas aspera*, with activity – guided repeated fractionation on silica gel column chromatography. Based on the various physico-chemical and spectroscopic analysis like UV, IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy, confirmed that the bioactive compound isolated was 5,7-dihydroxy-2-[14-methoxy-15-propyl phenyl]-4H-chromen-4-one (Fig. 1a) and termed as Leucasin [3]. A stock solution of 1 mg/ml of leucasin was prepared and stored for further experiments.

Bacteria

S. aureus ATCC 12600 was sub-cultured and maintained on nutrient agar. Bacterial cells in exponential growth phase, prepared in nutrient broth were harvested, washed and resuspended in 0.9% (w/v) sodium chloride [11].

Determination of MICs

The *MICs* of leucasin, novobiocin - the bacteriostatic antibiotic and penicillin G - the bactericidal antibiotic against *S. aureus* were determined by broth dilution assay with an inoculum density of ~5×10^5 cfu/ml [11].

Determination of *S. aureus* cells for loss of viability

A sterile 100 ml flask containing 20 ml of 20 mM phosphate buffer pH 7.0 with 0.625% (w/v) BSA and 1% (v/v) DMSO. A second flask containing 20 ml of 35 µg/ml leucasin was then prepared. Pure DMSO was employed as the flavone solvent and this was diluted to a final concentration of 1% (v/v) with 20 mM sodium phosphate buffer containing 0.625% (w/v) BSA. The two flasks were pre-warmed to 37 °C by placing them in an orbital incubator at 100 rpm. These were then inoculated with ~5×10^7 cfu/ml of *S. aureus.* After 0, 1, 2, 3, 4, 6, 8 and 12 h incubation, 0.1 ml samples were taken and viable counts were taken.

Examination of morphology of *S. aureus* cells treated with leucasin under SEM

SEM was done according to Zameer et al. [12] with several modifications. Briefly, 200µl of 5,000-fold diluted (~5×10^6 cfu/ml) bacterial suspension were evenly plated onto the membranes and the dishes were incubated upright for 24 h at 37 °C. Membranes with the *S. aureus* cells were treated with 35 µg/ml of leucasin for 1 h. Then the membrane was rinsed with phosphate buffer saline for 15-30 s and fixed for 12 h in 50 mM phosphate buffer, containing 6.25% (w/v) glutaraldehyde. Samples were dehydrated with acetone, subjected to critical point drying, coated for 300 sec with gold palladium and inspected using a Zeiss DSM 962 SEM (Carl Zeiss, Oberkochen, Germany). Images were captured at low and high magnifying cation to show details of 3D-architecture.

Effect of leucasin, novobiocin and penicillin G on potassium loss from *S. aureus* cells

Potassium loss assay was done according to Cushnie and Lamb [13] with few modifications. *S. aureus* of ~1×10^9 cfu/ml were incubated at 37 °C at 100 rpm in aqueous solutions of 30 ml 0.625% (w/v) BSA and 1% (v/v) DMSO which is taken as control. 35 µg/ml of leucasin, 0.625% (w/v) BSA and 1% (v/v) DMSO as treated cells to determine the potassium loss by flame atomic emission spectrophotometry (Model AA3100; Perkin-Elmer Ltd).

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis was performed using one way ANOVA followed by Duncan's multiple comparison test. Data was computed for statistical analysis by using SPSS statistical software. Statistical significance value was set at $p<0.05$.

Results & Discussion

In the present study, the possibility that leucasin exerts its antibacterial effect by damaging the cell membrane was investigated by measuring potassium loss from flavone-treated and untreated populations of *S. aureus*. In order to accurately quantify this potassium loss, it was necessary to be able to work with bacteria containing negligible quantities of background potassium. Leucasin is a novel compound exhibiting an interesting antimicrobial activity against *S. aureus*. This is the first report on antibacterial activity of leucasin and hence a very limited knowledge about mechanism of their action and spectrum of sensitivity to bacterial species is known. *S. aureus* was sensitive to leucasin at MIC 35 µg/ml to that of MICs of novobiocin and penicillin G against *S. aureus* are 55 ng/ml and 40 ng/ml respectively. Cells suspended in 35 µg/ml leucasin showed 100 fold decrease in viability (4.2×10^2 cfu/ml) compared to control cells (3.59×10^8 cfu/ml), while the viability of untreated cells remained stable.

Fig. 1a

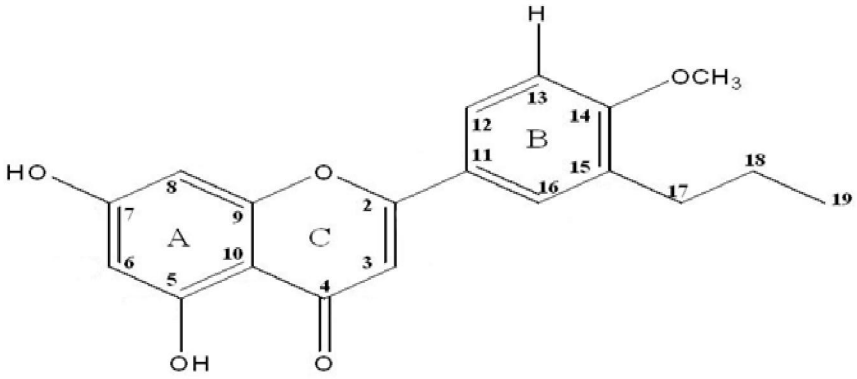

Fig. 1b

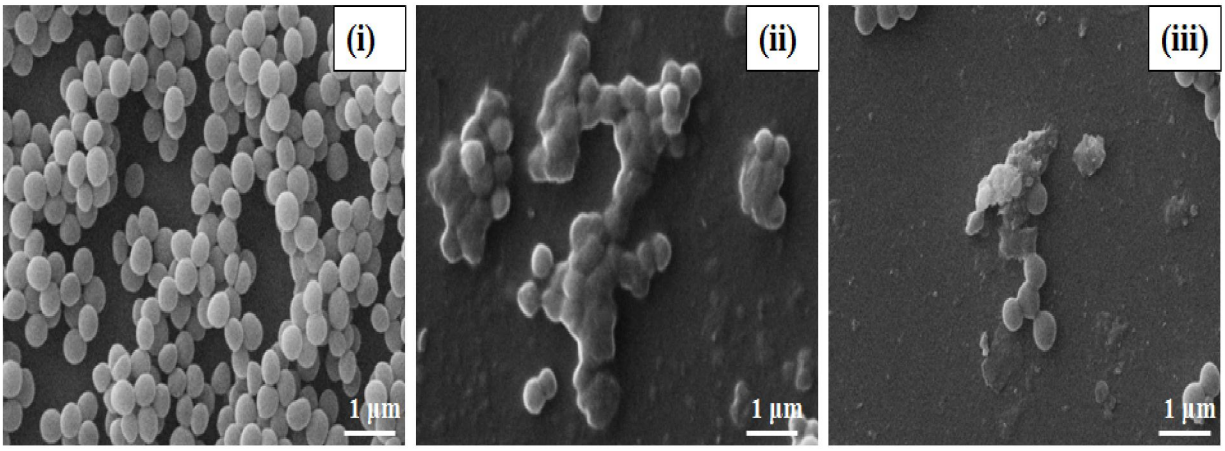

Fig. 1 a. Structure of Leucasin isolated from *Leucas aspera* leaves; b. Scanning Electron micrograph showing the morphology of *S. aureus* (i) Control cells, (ii) Leucasin treated cells and (iii) Penicillin G treated.

Leucasin inhibited/impaired the growth of *S. aureus* cells and the morphology was observed under SEM in the presence and absence of leucasin. However, the addition of leucasin at 35 µg/ml on *S. aureus* was found to show mucilaginous mass of cells which could lead to the impairment in cell division and chromosome replication (Fig. 1b, ii). However, the penicillin G showed the disruption of the cell membrane (Fig. 1b, iii) when compared to control cells remained stable (Fig. 1b, i). Bacteriostatic antibiotic novobiocin did not show much difference in the morphology of the *S. aureus* cells since even after the removal of leucasin *S. aureus* cells were able to regain their growth in the medium (data not shown). From the results of SEM, it was evident that leucasin exerted its antibacterial effect, potassium loss was investigated to check whether leucasin could probably have a role in cytoplasmic membrane damage. It was observed that, inspite of making careful adjustments to media composition, the amount of potassium loss from cells of *S. aureus* was quite high (Fig. 2). This is probably a consequence of autolysis which occurs when cells are exposed to antibiotics or stressful physiological conditions [14]. In Fig. 2a, it was observed that leucasin has a substantial effect on *S. aureus* with leucasin-treated cells losing 17% more potassium than untreated control cells. This effect has occurred as a result of leucasin-induced potassium efflux. In general, when bacterial cells are exposed to toxic compounds, can release potassium in order to acidify the cytoplasm and minimize the rate at which lethal lesions are formed [15]. It is also possible that the results in Fig. 2a were caused by leucasin inhibiting potassium uptake. In the potassium loss assay, flavone-treated and untreated cells are both likely to be losing potassium through starvation-induced autolysis, and the structural similarity between leucasin and ATP may allow the flavonol to interfere with Kdp and Trk influx systems. Decrease in the viability of leucasin treated *S. aureus* cells substantiates that leucasin induces cytoplasmic membrane damage and thus potassium leakage. This does not necessarily mean that leucasin

is damaging the cell membrane directly. It may be that the leucasin exerts its effect elsewhere in the cell and this event triggers secondary autolysis. In order to investigate these possibilities further, the potassium loss profiles of other antibacterial agents with known mechanisms of action were examined.

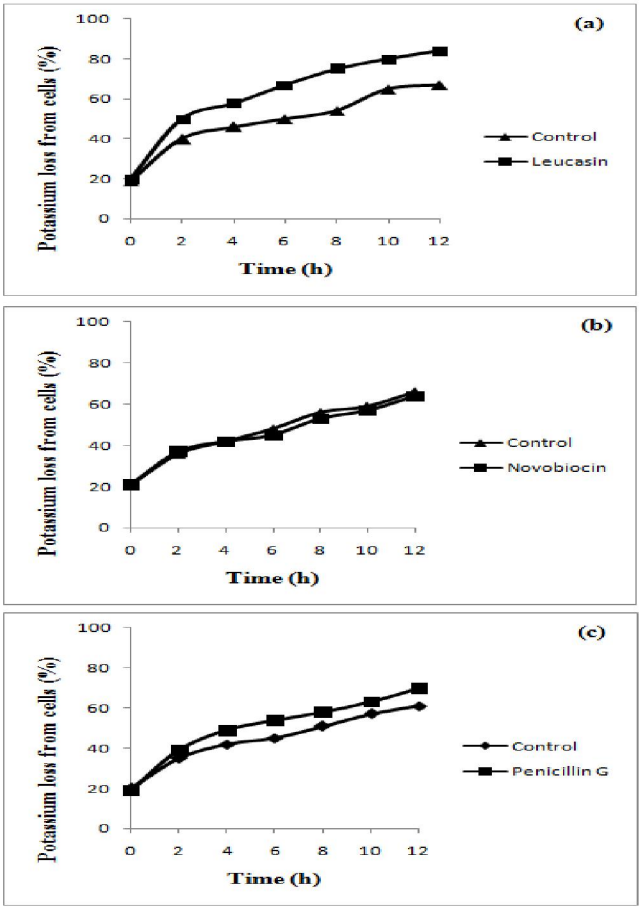

Fig. 2 Percentage potassium loss of *S. aureus* treated with **(a)** 35 µg/ml leucasin; **(b)** 55 ng/ml novobiocin and **(c)** 40 ng/ml penicillin G

In Fig. 2b, novobiocin incubated with the cells of *S. aureus*, no potassium loss was observed when compared to untreated control cells. These results are not surprising as this antibiotic is known to exert its antibacterial effect not through membrane damage, but by inhibition of type II DNA topoisomerase enzymes. Also, novobiocin is reported to be bacteriostatic agent [16], so potassium loss was not expected to occur through antibiotic-induced autolysis. Interestingly though, the results indicate that potassium was not released or pumped out of bacterial cells in response to the presence of this antibacterial agent. The similarity in the levels of potassium loss from treated and untreated cells also indicates that, despite being partially analogous to ATP [17], novobiocin does not inhibit potassium influx. This data represents further evidence that potassium loss observed from leucasin-treated cells (Fig. 2a) occurred, not through efflux or inhibition of influx, but as a result of membrane damage.

In Fig. 2c, *S. aureus* cells treated with penicillin G, lost 9% potassium than control cells. As β-lactam antibiotics exert their activity through inhibition of cell wall synthesis, rather than membrane damage, lysis of bacterial cells is commonly associated with these agents. It is probable that this is partially due to the β-lactam compounds reduces the mechanical strength of the cell wall, with lysis occurring as a result of the effects of osmotic pressure [18]. This may explain the data presented in the Fig. 2c. if the osmolarity of 0.625% (w/v) BSA and 1% (v/v) DMSO solution is slightly low, water could be entering *S. aureus* cells by osmosis, and bacteria with penicillin G weakened cell walls may be bursting under the increased pressure. Although, potassium loss induced by leucasin (Fig. 2a) 5 times greater than that observed with penicillin G (Fig. 2c), the possibility that leucasin's antibacterial mechanism of action also involves damage to the cell wall or inhibition of its synthesis cannot be excluded. In addition to osmotic lysis, there is accumulating evidence to indicate that β-lactam antibiotics are known to kill bacteria by triggering autolysis. This is an equally valid explanation for the penicillin G-induced potassium loss observed in Fig. 2c. It remains possible, that potassium loss induced by leucasin occurred through indirect membrane damage caused by autolysis.

The observation of increased potassium loss in treated cells is very interesting and represents a useful lead worthy of further investigation. Leucasin may be damaging the cytoplasmic membrane directly or weakening the cell wall and thereby causing osmotic lysis [19]. However, new classes of antimicrobial drug are urgently required and the flavonoids represent a novel set of leads. Hence leucasin could be attributed as a potential tool for the prevention of *S. aureus* related infections and diseases.

Acknowledgement

Dr. S. Gopal thanks Department of Science and Technology, New Delhi for their financial assistance.

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