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Effect of Lupeol, A Triperpenoid Compound on the Drug Resistant Gene, MecA of Methicillin Resistant *Staphylococcus Aureus*

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Abstract

mec A gene is found to be responsible for drug resistance in Methicillin Resistant *Staphylococcus aureus*. Lupeol obtained from aqueous extract of bark of *Alstonia scholaris*, a traditional medicinal plant inhibited the growth of MRSA. The aqueous extract obtained from the bark of the tree analyzed by LC-MS and found to contain lupeol. On treating with MRSA cultures, it is found to have an inhibitory action at concentration of 50µg/ml. On RT-PCR analysis and mec A expression protein analysis, it is revealed that lupeol inhibits cell wall synthesis at sub inhibitory concentration of 20µg/ml when added in the growth medium. It inhibits mec A gene.

Keywords: MRSA; Lupeol; Alstonia scholaris; RT-PCR, MRSA; MecA

Introduction

Emergence of drug resistance is an alarming threat to the medical field. Microbes develop resistance due the regular use antibiotics. Methicillin Resistant Staphylococcus aureus (MRSA) is one such organisms. There are different mechanisms which causes the development of drug resistance. mecA is the gene associated with drug resistance in MRSA [1,2]. Plant antimicrobials are a cheap alternative against MDR. The gene responsible for different drug resistance have been reported in S. aureus [3-5]. Lupeol is a triterpenoid compound found in vegetables like cabbage, tomato etc. It is an anti-inflammatory agent and can affect the pathways having nuclear factor kappa B (NFκB), cFLIP, Fas, Kras, phosphatidylinositol-3-kinase (PI3K)/Akt and Wnt/β-catenin in a variety of cells. The action of Lupeol obtained from the extract bark of Alstonia scholaris was tested against the MRSA in the present study [6].

Materials and Methods

Plant material was selected locally and identified and deposited in the herbarium, Department of Botany, University of Calicut (Voucher no.6234). Extract was prepared as described earlier with some modifications. In brief the bark collected was air dried and extracted with sterile water [7].10 gm of the sample was mixed with 50 of sterile water and kept on the shaker at 200rpm for 10 hrs. The supernatant was collected and filtered and dried. The extract was subjected to bioassay guided fractionation with increasing order of polarity, starting from solvent with least polarity. Solvents such as hexane, chloroform, methanol and water were used for extraction. The final extract with water has subjected to dryness. The dry mass was then dissolved to get a extract of concentration 1mg/ml and stored under refrigeration as stock till further use. MRSA (ATTC 1109) strain was procured from the Jubilee hospital, Thrissur used for the study. The culture was screened with oxacillin at 5µg/ml concentration and screened for resistance using nutrient agar plates using Bauer Kirby method and as per the NCCLS, 2000.

For RT-PCR study MRSA broth were prepared in Muller-Hinton medium with the extract of concentration 25µg/ml. The experiment was done with PCR primers as reported by Mariana et al, [8] with some modifications [8]. Total RNA was extracted from cells in the logarithmic phase of growth and purified using Guanidium Isothiocyanate. Unless otherwise specified, cells were grown to mid logarithmic phase in NB (pH 6.6) at 36°C, conditions optimum for expression of the toxic proteins. 750µl culture was centrifuged and resuspended in 600µl Lysis buffer (freshly supplemented with 0.7% β-ME) and mixed well by vortex. 60µl of 2M Na-acetate (pH-4.0) was added and mixed with vortex. An equal volume of hot phenol (68°C) saturated with DEPC water (pH 4.0) was added and vortex vigorously for 5 minutes. The mixture was incubated at 68°C for 10 minutes, cooled, 120µl of chloroform

was added and vortex vigorously for 15 minutes with intermittent incubation on ice.

The mixture was centrifuged, 150µl of the aqueous phase was transferred to a fresh micro-centrifuge tube and equal volume of isopropanol was added. The solutions were mixed well and incubated at -20C for 1-2 hours. RNA was precipitated by centrifugation at 13,000 rpm for 20 minutes and the pellet was dissolved in 500µl of Lysis solution. RNA was re-precipitated by adding an equal volume of isopropanol, kept at -20°C for 1-2 hrs. After centrifugation, the pellet was washed in 80% ethanol, dried at room temperature and dissolved in 10µl of DEPC treated water. RNA was quantified by measuring the absorbance at 260nm (A)*. For all experiments with RNA, extensive precautions against RNase contamination were taken.

Semi-quantitative RT-PCR

Reverse transcription of the isolated RNA was performed to synthesize the first strand of cDNA with reverse primer and then amplification of cDNA was done using specific Primer sets.

Procedure

Isolated RNA samples were subjected to DNase treatment to make them free from any contaminating DNA.

a. For cDNA synthesis a total of 200ng of RNA was taken, incubated at 70°C with specific anti sense primer for 10 minutes annealing in a thermal cycler.

b. Add 5X buffer, and then Superscript RT at 42°C, allow the reaction to carry in thermal cycler for successful reverse transcription.

c. Following cDNA synthesis, amplification of specific genes responsible for cell wall synthesis (mecA genes) was done using specific primer sets.

d. Amplification was for 35 cycles (each cycle consisted of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, followed by a seven-minute extension at 72°C)

e. Genomic DNA served as a positive control, and DNase treated RNA that had not been reverse transcribed was used as a negative control.

f. Aliquots removed at 25, 30 and 35cycles for each PCR product was electrophoresed, and the gels were analyzed with a Gel Doc System.

g. PCR products were normalized according to the amount of 16S rRNA detected in the same cDNA sample.16S rRNA is a housekeeping gene and is constitutively expressed.

h. Each set of experiments was repeated at least thrice.

DNase Experiment Set Up

DNase treatment of isolated RNA samples were done before cDNA preparation to remove any DNA contamination with RNA, so that Reverse Transcriptase can only reverse transcribes the mRNA to prepare the complementary DNA.

a. The procedure generally follows the given set up:

b. Autoclaved water -- As required to make the total volume 10µl.

c. RNase Inhibitor -- 0.5µl

d. RNA -- Desired volume in microliter to have total 1micogram

e. DNase Enzyme -- 1µl

f. (Total 10µl of reaction set up)

g. This reaction mixture was kept for 30 minutes at 37°C water bath. The reaction was stopped by adding 1micoliter EDTA to each microfuge tube, to chelate Mg2+ ions. Finally, the heat inactivation of DNase enzyme was done at 70°C water bath for 10 minutes.

LC-MS analysis of the extract

5µl of the sample was injected to the HPLC. The system was with dual pump, rhedyne injector SPD photodiode array detector and 6.12 sp5 integration software. The compound was identified as lupeol by LC MS analysis [9].

Discussion

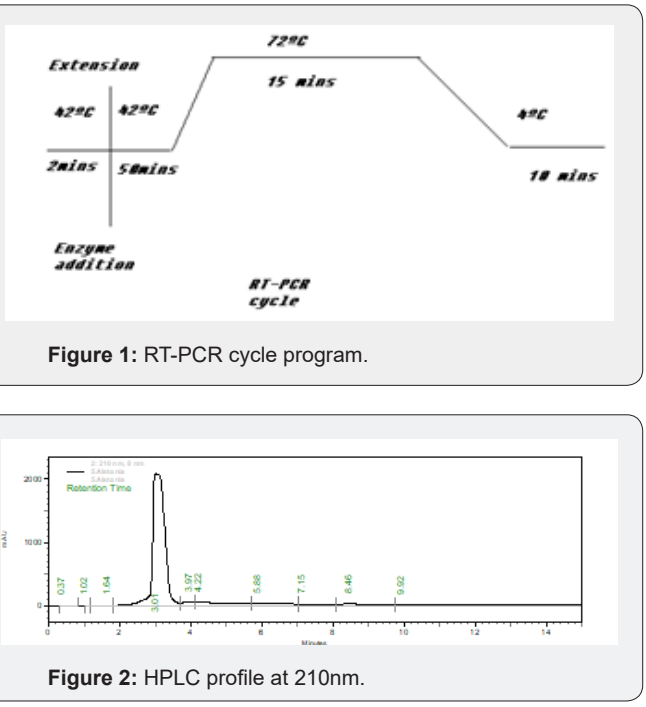

Figure 1: RT-PCR cycle program.

Figure 2: HPLC profile at 210nm.

LC-MS analysis showed the presence of lupeol in the extract (Figures 1-3). PCR results clearly indicate that lupeol inhibits the mecA synthesis in MRSA at mRNA level (Figure 4 & 5). Lupeol can be used to develop a new drug against MRSA infections. Use of antimicrobials against drug resistance was described earlier by different workers [10-12]. Earlier research showed that lupeol is

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a strong anticancer agent. The action mechanism against amino acid synthesis has to be studied for further verification of the result.

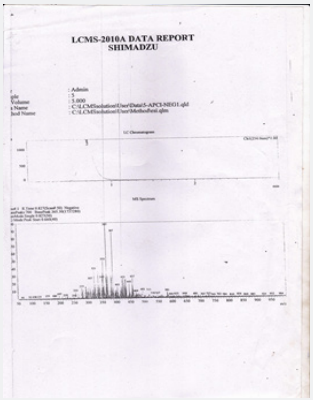
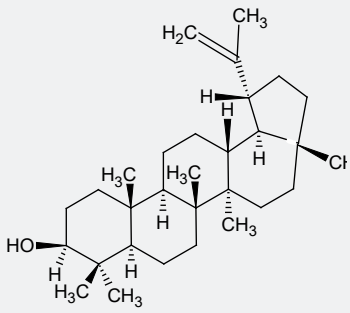


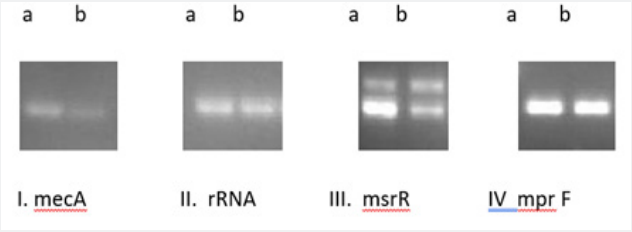
Figure 3: LC-MS analysis of A.scholaris extract.

Lupeol



Molecular Formula = $C_{31}H_{54}O$
Formula Weight = 442.75986
Composition = C(84.09%) H(12.29%) O(3.61%)
Molar Refractivity = 138.48 ± 0.3 cm^3
Molar Volume = 472.2 ± 3.0 cm^3
Parachor = 1129.3 ± 6.0 cm^3
Index of Refraction = 1.498 ± 0.02
Surface Tension = 32.7 ± 3.0 dyne/cm
Density = 0.937 ± 0.06 g/cm^3
Dielectric Constant = Not available
Polarizability = 54.89 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass = 442.417466 Da
Nominal Mass = 442 Da
Average Mass = 442.7599 Da
M+ = 442.416918 Da
M- = 442.418015 Da
[M+H]+ = 443.424743 Da
[M+H]- = 443.42584 Da
[M-H]+ = 441.409093 Da
[M-H]- = 441.41019 Da

Figure 4: Lupeol



i. Control with no lupeol in the medium b. medium with lupeol(20µg/ml) amplified with mecA primers.

ii. Control with no lupeol in the medium b. medium with lupeol(20µg/ml) amplified with rRNA primers.

iii. Control with no lupeol in the medium b. medium with lupeol (20µg/ml) amplified with msrR primers.

iv. Control with no lupeol in the medium b. medium with lupeol (20µg/ml) amplified with MPR F primers.

Figure 5: RT-PCR analysis of MRSA for the study of action of lupeol at sub inhibitory concentration of 20µg/ml of lupeol.

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