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Effects of Sub-MIC of Rifaximin and Essential Oils on Biofilm Formation and Invasion of *Staphylococcus aureus* Isolated from Mastitis

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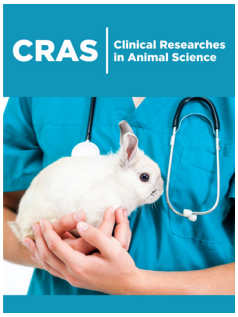

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Significance and Impact of the Study

Mastitis can be caused by *Staphylococcus aureus*, which is hard to be eradicated, especially when it formed biofilm. This situation has made searching for novel antimicrobial agents urgent. Present study demonstrates the anti-bacteria and anti-biofilm inhibitory activity of essential oils on *S. aureus* alone and combined with rifaximin. Combinations of rifaximin and mint oil can not only inhibit *S. aureus* from growing on abiotic material, but also restrain its invasion into cells. Results of the present study gave an insight into the promising alternatives for killing *S. aureus* and treating mastitis.

Abstract

This study intended to explore whether rifaximin and EOs (mint oil (MEO), eucalyptus oil (EEO), and EO of Houttuynia cordata (HEO)) alone and combined can inhibit the biofilm formation and invasion ability of *S. aureus*. Firstly, minimal inhibitory concentration (MIC) of 9 strains were investigated. Otherwise, checkerboard microdilution assays were used to measure FICI of rifaximin and EOs of *S. aureus*- 2. Lastly, the influence of the sub-MIC of EOs alone and combined with rifaximin on the biofilm formation and invasion of *S. aureus* were tested. MEO exhibited the greatest efficacy against planktonic cells. The results demonstrated that combination of rifaximin and MEO was additive, and the others showed indifferent. Almost all EOs alone and combined with rifaximin effectively reduced the *S. aureus* biofilm formation to some extent. Meanwhile, combination of rifaximin and MEO showed the strong inhibitory effect on invasion, and 0.5MIC rifaximin +0.125MIC MEO can inhibit bacteria from invasion. These results suggested rifaximin alone and combined with EOs, in particular MEO, can be the alternative therapies for bovine mastitis.

Keywords: *Staphylococcus aureus*; Invasion; Rifaximin; Biofilm; Essential oils; Combination

Introduction

Bovine mastitis is the inflammation of the bovine mammary glands, leading to numerous economic losses, such as reduction of the milk production, waste of substandard milk, etc [1]. Bacteria are major causes of mastitis, of which *Staphylococcus aureus* (*S. aureus*) is an important one [2]. The most common treatment of mastitis is antibiotic therapy, however, cure rates for *S. aureus* mastitis are variable, ranging from 4 to 92% [3]. There are studies showed that an important reason for reduced therapy success rates is resistance of *S. aureus* [4]. The increasing prevalence of antibiotic resistance of *S. aureus* may contribute to biofilm-forming abilities [5].

Biofilm of *S. aureus* is a complex microbial community embedded in a self-produced extracellular matrix on abiotic surfaces or animal tissues, such as bovine utter [5-7]. The adhesion and invasion ability of *S. aureus* may be one of the foundations of biofilm establishment [8]. The matrix can act as a protective layer for bacteria, allowing bacteria

to be more resistant to antibiotics, eventually making antibiotics treatment low efficiency [5,9,10].

Current situation has made search for alternative strategies for combating biofilm necessary. Rifaximin is intended for prevention and treatment for mastitis during the dry period, but there were limited articles about its influence on biofilm. EOs, which are natural antimicrobials, may be potential therapy to combat biofilm [11]. Moreover, EOs can also inhibit *S. aureus* from invasion, such as citrus-derived oil [12]. Otherwise, combination therapy can be a promising alternative in that it may extend spectrum coverage, prevent resistant mutants from emergence and gain synergy between drugs [13]. What's more, hydrophobic feature of EOs may render cells more permeable to the uptake of antibiotics [14]. Which is to say, the combinations of EOs and traditional antibiotics may be available treatment approaches.

Based on above, trails had been carried out to discover whether rifaximin and EOs alone and combined can inhibit biofilm-formation, and co-culture model was established to figure out the effect of the rifaximin and MEO on adhesion and invasion of *S. aureus* into MAC-T.

Result and Discussion

Antibacterial activity of rifaximin and EOs alone and combined

The MICs of antibiotics and EOs against *S. aureus* in suspension were shown in Table 1. Some MIC were over 5%, HEO at the concentration of 2.5% failed to kill most of the stains of our experiment, while EEO was able to kill most of the bacteria at the same concentration, as for MEO, the MIC of that needed to eradicate cells in suspension was below or equal to 2.5%. Which is to say, MEO showed the highest inhibitory effects on planktonic cells of all strains tested.

Table 1: MIC of essential oils.

(%, vol/vol)	S.a-1	S.a-2	S.a-3	S.a-4	S.a-5	S.a-6	S.a-7	S.a-8	S.a-9
EEO	1.25	2.5	5	5	0.625	1.25	5	2.5	2.5
HEO	5	1.25	>5	5	5	5	2.5	2.5	2.5
MEO	1.25	0.17	2.5	1.25	2.5	0.625	1.25	0.31	0.17

S.a represents for S.aureus.

As Table 2 showed, rifaximin in combination with MEO showed an additive effect (0.5<FICI≤1), the rest two combinations had an indifferent effect (1<FICI≤2). There were reports indicating that the hydrophobicity of EOs may render cell membrane more permeable to the uptake of antibiotics, and components of essential oils as thymol and carvacrol can have same effect, too [15,16]. But the naturally derived antimicrobials have more biochemical and structural diversity than common drugs [10].

Table 2: The FICI of Rifaximin and EOs against *S. aureus*.

Agent A (μg-ml^{-1})	Agent B (% vol/vol)	MIC of Agent A		MIC of Agent B		FICI	Effect
		Alone	Combination	Alone	Combination		
RIF	EEO	0.0156	0.0156	1.25	0.625	1.5	Indifferent
	HEO	0.0156	0.0039	5	5	1.25	Indifferent
	MEO	0.0156	0.0039	0.1563	0.078	0.75	Additive

Anti-biofilm activity of rifaximin and EOs alone and combined

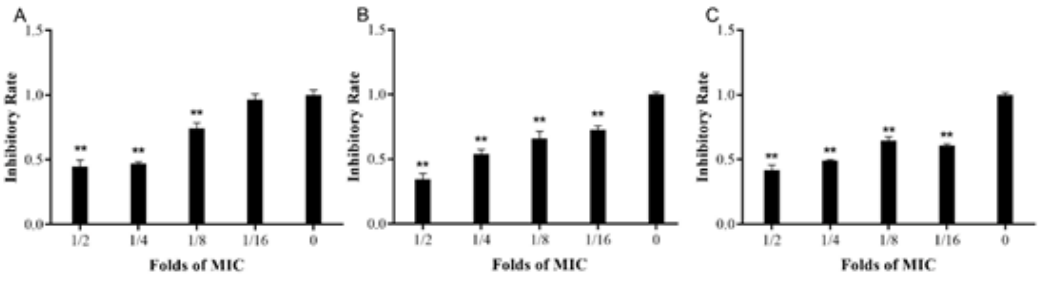

Figure 1: Effects of EOs at sub-MIC on *S.aureus* biofilm formation.
The biofilms were stained with crystal violet and the optical density was determined with a multi-detection microplate reader of OD595nm. Data shown are representative three independent experiments and are expressed as mean ±SD.
A. EEO treatment group.
B. HEO treatment group.
C. MEO treatment group.
*Represent statistical significance (P≤0.05), **Represent extreme significance (P≤0.01).

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The influence of sub-MIC of different kinds of drugs on the biofilm formation of *S. aureus*-2 was determined through CV staining. Results were shown in Figure 1. When treated with sub-MIC of HEO and EEO, inhibition was stronger with the concentration of drugs increasing. HEO at all concentrations exhibited an extreme significant inhibitory effect on biofilm formation compared to the control (P≤0.01). The biofilm treated with EEO at concentration of 0.063MIC had no difference from the control (P>0.05), and other concentrations were different from the control (P≤0.05). There was extreme significance between treatment group of MEO with the control group (P≤0.01). But treatment of 0.125MIC of MEO had stronger inhibition effect than adjacent group, with no difference showed.

It's been studied that whether the treatment of established biofilms with combinations of rifaximin and EOs would reduce biofilm biomass more than treatment with EOs alone. As the Figure 2 showed, the combination of 0.5MIC rifaximin+0.5MIC MEO can inhibit the biofilm-forming most effectively, with inhibitory rate of 90%, the combination of 0.5MIC rifaximin+0.5MIC EEO reduced biofilm biomass by 55%, and the combination of 0.5MIC rifaximin+0.5MIC HEO gave a 60% reduction. The combination of drugs whose concentrations are both 0.063MIC shows no difference with the control group.

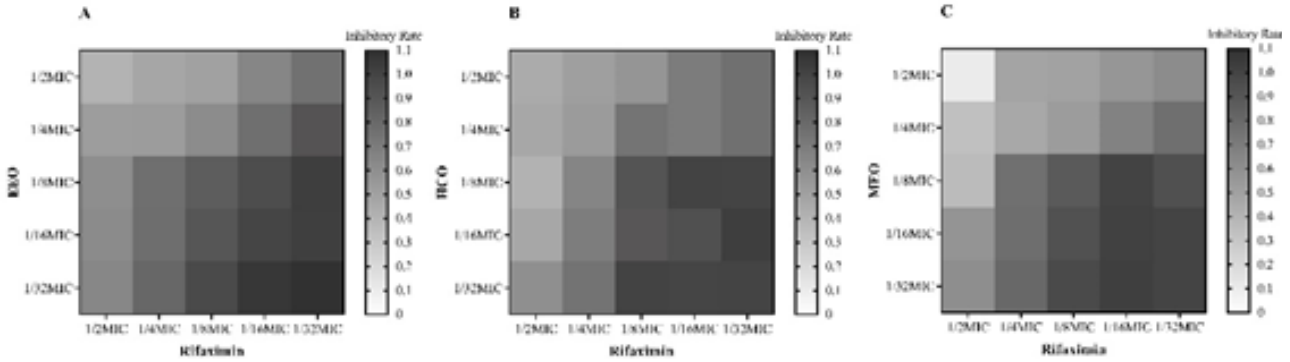

Figure 2: Effects of rifaximin and MEO at sub-MIC on *S.aureus* biofilm formation.
The biofilms were stained with crystal violet and the optical density was determined with a multi-detection microplate reader of OD595nm. Data shown are representative three independent experiments and are expressed as mean ±SD.

A. EEO treatment group.
B. HEO treatment group.
C. MEO treatment group.

EOs possess antimicrobial and anti-biofilm activity with the potential for use as therapeutic agents [17]. MEO, which belonged mint family, is rich in cavracrol and thymol have a high efficacy against pathogens bacteria [18]. EEO can inhibit biofilm of Staphylococcus epidermidis, E. coli, and Salmonella paratyphi, is a promising agent to be applied to combat biofilm [19]. Extracts of H. cordata showed to be anti-bacterial and anti-biofilm, but the effect of EOs on biofilm are not studied yet [20-22].

Combination of rifaximin and EOs showed stronger anti-biofilm ability than drugs using alone at certain concentrations. However, there are little article involved the combined effect of rifamycins and EOs. Rifampin, the analog of rifaximin, is effective alone or in combination with other antibiotics against the bacteria forming biofilms [23]. Combination can be useful application, since there may be a great chance that bacteria seldom produce monotherapies [14,24]. It's showed that antibiotics of the cell-wall active class can be combined with rifampin [17]. What's more, EOs may be able to penetrate on the cell wall of Gram-positive bacteria, in that cell structure of gram-positive bacteria and hydrophobic features of Eos [17,25]. The reports showed that EOs combined with its composition may be more effective than using alone, S. aureus showed malformed cell surface or broken cells with pores formation [26]. The combination of rifaximin and MEO was more effective may be related to the composition, such as mint family, which is rich in cavracrol and thymol [18].

Cytotoxicity of rifaximin and MEO on MAC-T cells

The results of drugs used on this study on MAC-T cells after 3h and 24h were showed in Figure 1. There was no significant cytotoxicity (P>0.05) observed between the group treated with rifaximin and gentamicin and the control group at 3h or 24h. The MEO at 0.125MIC was not significantly different (P>0.05) with the control, however, at the higher concentrations (0.25MIC), extreme significance was observed (P<0.01) at 3h, and significant difference was observed (P<0.05) at 24h. Above all, 0.5MIC RIF and 0.125MIC MEO were chosen for further study.

Effect of rifaximin and MEO on *S. aureus* invasion into MAC-T

0.5 MIC Rifaximin+0.125 MIC MEO, the non-toxic group, was chosen for invasion assay. The results were shown in Figure 3, showing rifaximin and MEO alone and combined has an effect on bacteria invasion. Invasion of *S. aureus* into MAC-T cells were reduced in 0.125 MIC MEO and 0.5 MIC Rifaximin groups, respectively, which is different (P≤0.05) and significantly different (P≤0.01) compared

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with the control group. The result showed that treated group of 0.5 MIC Rifaximin+0.125 MIC MEO was significantly different (P≤0.01) compared with the control group, 0.5 MIC Rifaximin group and 0.125 MIC MEO group. The effect of drugs alone and combined on invasion confirmed that rifaximin and MEO can inhibit *S. aureus* from attaching to biotic and abiotic material, and the effect of drugs combined was more effective than alone. There were no reports about MEO's effect on invasion to our knowledge, not to mention the articles about combination of antibiotics and EOs. Intramammary bacterial adherence is an important factor of biofilm formation, at the same time, the biofilm formed appears to enhance binding of *S. aureus* to epithelial cells [27]. Mammary epithelial cells are vital for surveillance of mammary tissue by recruiting immune cell and recognizing bacterial during infection [28]. Invasion of *S. aureus* into bovine mammary epithelial cells was related to surface-associated proteins, further study is required to find out whether MEO effecting biofilm-related genes and surface-associated proteins [29].

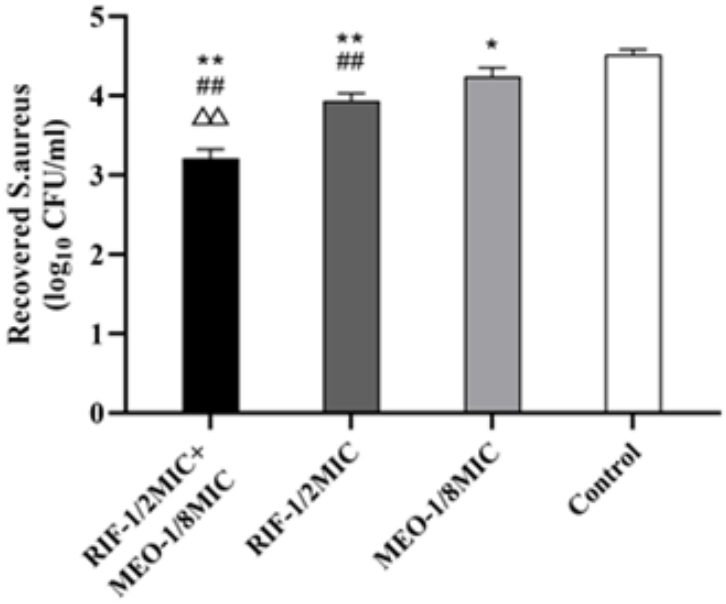

Figure 3: Effect of rifaximin and MEO on *S.aureus* invasion into MAC-T.
A co-culture group with no drug added was used a control. *Represent statistical significance with control group (P≤0.05), **Represent extreme significance (P≤0.01); #Represent statistical significance with MEO group (P≤0.05), ##Represent extreme significance (P≤0.01); △Represent statistical significance with RIF group (P≤0.05), △△Represent extreme significance (P≤0.01).

Material and Methods

Bacterial isolates and culture conditions

Nine Staphylococcus aureus (*S. aureus*) isolates were obtained from milk acquired from bovine suffered from bovine mastitis in a dairy farm at Pingdingshan, Henan, China. *S. aureus*-2 was choosing for further study for its strong biofilm-forming ability. The isolate was cultured in TSB at 37 ℃ for 8-10h (mid-log phase) for further study.

Mammalian cell culture

Bovine mammary alveolar cell line (MAC-T, BNCC338264, BNBIO, Beijing, China) was used for invasion assays. MAC-T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, USA) at an incubator with 5% (vol/vol) CO_2.

Antimicrobial agents and essential oils

Rifaximin was purchased from National Institutes for Food and Drug Control (Beijing, China). Three EOs, MEO, HEO and EEO were buying from Huayuan Perfume Company. Stock solutions (1280μg·ml^{-1}) of rifaximin were prepared in dimethyl sulfoxide (DMSO), sterile filtered (0.22-μm pore diameter) and stored at -20 ℃ for 2 weeks. Stock solutions at concentration of 50% EOs were solubilized with equal amount of ethanol plus Tween-80 and dissolved in TSB to produce solution of 10% used for further study.

MIC

MIC for EOs and antibiotics was determined by the microdilution methods according to Clinical and Laboratory Standards Institute [30]. Briefly, culture in mid-log phase was diluted to 106CFU·ml^{-1} and 100μl suspension was then added into wells supplemented with 100μl EOs solution which is serially two-fold diluted. After incubation overnight, MIC was determined as the lowest concentration preventing growth.

FIC assay by the checkerboard method

Combination susceptibility of rifaximin and EOs was tested by the checkerboard micro-dilution method. Rifaximin and EOs were serial two-fold dilutions in two 96-well microtiter plates with final concentration ranging from 16 to 0.5-fold of the MIC. Then, 50μl of

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rifaximin and 50μl of EOs serial dilutions were added horizontally and vertically into the plates, 100μl of inoculates (106CFU·ml^{-1}) were added into all wells, and incubated at 37 °C for 24h. The formulas used to calculate the FICI are as followed: FICI=FICIA+ FICIB=MICA Combined/MICA alone + MICB Combined/MICB alone. The result was used to explain the effects of combination susceptibility of the drugs: synergy (FICI≤0.5), interaction (0.5<FICI≤1), indifference (1< FICI≤2), and antagonism (FICI>2). The tests were performed in three replicates.

Biofilm production at sub-MIC of rifaximin and EOs alone or combined

Biofilm formation in the presence of sub-inhibitory rifaximin and EOs concentrations (0.5MIC-0.063MIC) was performed according to the method previously [31], with modifications. *S. aureus* culture (100μl, 106 CFU·ml^{-1}) was added to 96-well microtiter plates, and drug solutions were added to attain a final concentration at 0.5MIC to 0.063MIC. TSB broth (100μl) mixed with 100μl of *S. aureus* culture was used as positive control, and TSB broth (200μl was used as negative control. The microplates were incubated at 37 °C for 48h, and biofilm production was dyeing with CV and taking readings at OD595nm. All tests were performed in three replicates.

Cytotoxicity of rifaximin and MEO alone or combined on MAC - T Cells

The 3 - (4,5- dimethylthiazol-2-yl) - 2,5-diphenyltetra zolium bromide (MTT) assay methods were applied for cytotoxicity of drugs on MAC-T. Epithelial cells (104 cells per well) were added to a 96-well plate at 37 °C, 5% CO_2 overnight. The medium was removed, and cells were washed 3 times. The plate was added with 200μl of DMEM supplemented with 10% FBS, rifaximin at 0.5MIC and MEO at 0.5MIC, 0.25MIC, or 0.125MIC alone or combined, and gentamicin at 100μg·ml^{-1}, and incubated for 3h at 37 °C, 5% CO_2. Non-treated cells and medium were positive and negative controls. Medium was removed again, and the plate was washed 3 times with DMEM before 200μl of DMEM supplemented with 20μl of 5mg·ml^{-1} of MTT was added. The cells were incubated for 4h, afterward, medium was removed and 150μl of DMSO was added to each well. OD490nm of the plate was tested after shaking for 10 to 15 min. 100% DMSO was used as blank. The tests were performed in triplicate.

Effect of rifaximin and MEO on *S. aureus* invasion into MAC-T

To quantify the adherent and invaded bacteria, an adhesion assay was performed according to Silva et al (Silva et al., 2014), with modifications. Briefly, *S. aureus* was grown in 5ml TSB overnight at 37 °C. Bacteria were centrifuged (5min, 5,000r·min^{-1}, 4 °C), washed twice three times with phosphate-buffered saline (PBS, pH 7.4; Solarbio), and resuspended in cell culture medium. Monolayers of MAC-T cells (105 cells per well) were cultured in 24-well plates (Falcon, Corning Inc., NC, USA). were washed three times with PBS and infected by *S. aureus* at a multiplicity of infection (MOI, the ratio of *S. aureus* to MAC-T) of 100. At the same time, drugs were added at a final concentration of 0.5 MIC RIF, 0.125 MIC of MEO, and 0.5 MIC RIF+0.125 MIC of MEO. After 3h of invasion, MAC-T monolayers were washed with PBS for three times, non-internalized bacteria were killed by gentamicin (100μg·ml^{-1}) for 2h. Monolayers were washed three times with PBS and lysed with 1ml of 0.25% (wt/vol) trypsin and 0.1% (vol/vol) Triton X-100 in PBS for 10 min to obtain the intracellular bacteria. Lysates were 10-fold serially diluted and cultured on MH agar at 37 °C for 24h. Control wells (without antimicrobials) were performed as above. Each assay was performed with triplicate samples.

Statistical analysis

A significance level of 5% (P>0.05) was used for statistical analyses. All data were presented as mean ± standard deviation. Statistical analysis was performed by using SPSS19.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's test was used for multiple comparisons.

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Conflict of Interest

No conflict of interest declared.

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