

# The *In-vitro* Antibacterial Effect of Colored Rice Crude Extracts against *Staphylococcus aureus* Associated with Skin and Soft-Tissue Infection

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## Abstract

Due to increases in the resistance of bacteria to the existing antimicrobial agents, plants are being used as alternative sources for the development of safe, effective, and inexpensive new agents to treat and prevent bacterial infections. Recent studies have shown that rice (*Oryza sativa* L.), an important source of nutrients, consumed by most of the world's population, can suppress some bacterial infections. There are many varieties of rice, e.g. white, brown, black, and red; however, the relationship between rice color and antibacterial properties remains unclear. In the present study, we investigated the antibacterial activity of colored-rice crude extracts from four different types of colored rice (Hom Nil, Neaw Dum, Mun Poo, and Sang Yod) against common bacteria causing skin and soft-tissue infections, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus* spp., *Pseudomonas aeruginosa*, and *Escherichia coli*. The results showed that all colored-rice crude extracts had antibacterial effects against *S. aureus*; and more notably, crude extracts of differently colored rice restricted diverse antibacterial activities. To our knowledge, this is the first report that provides a basic understanding of the antibacterial properties of colored rice against skin and wound pathogens. An understanding of these properties would be invaluable in the development of alternative, natural, and safe methods of controlling bacterial infections.

**Keywords:** *Oryza sativa* L., colored rice, antibacterial effect, skin and soft tissue infections

## 1. Introduction

Bacterial skin and soft tissue infections (SSTIs) are some of the most common infections that can occur throughout the human life span (Compton, 2013; Ki & Rotstein, 2008; Sarlangue, Boralevi, Barba, & Leaute-Labreze, 2001). The clinical manifestations of SSTI range from simple uncomplicated superficial/cutaneous skin infections, such as cellulitis, erysipelas, impetigo, abscess, and wound infections, to life-threatening conditions, such as necrotizing fasciitis (flesh-eating bacteria) (Ki & Rotstein, 2008; Tognetti et al., 2012). Bacterial SSTIs can be caused by both Gram-positive and Gram-negative organisms (Doern, Jones, Pfaller, Kugler, & Beach, 1999; Khokhlova, Karelin, Belotserkovskii, & Stetsiuk, 2011), with the most common causative agents being Gram-positive *Staphylococcus aureus*, *Streptococcus pyogenes* and *Enterococcus* species. SSTIs are also caused by a variety of Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Escherichia coli*. Most infections are self-limited, but some complicated conditions require antibiotic treatment and/or hospitalization. In recent years, these infections have become more difficult to treat, as pathogens have developed resistance to many different types of antibiotics (Jones et al., 2004; Liu, 1999). While drugs to treat bacterial SSTIs are being investigated developed and tested, documented cases of treatment failure and adverse side effects (Sacchidanand et al., 2005; Scheinfeld, 2005; Upadya & Ruxana, 2009) have added urgency to the development of new prevention and treatment modalities.

There is growing interest in plant-derived substances as alternative antimicrobial agents for the prophylaxis and treatment of disease (Mahady, 2005; Martin & Ernst, 2003). The advantage of using plant medicine is that it is a natural product, and normally safer than synthetic or chemical compounds. Rice (*Oryza sativa* L.) is one of the most common plant products consumed by humans. It is grown worldwide and in some countries is part of almost every meal. It is known that rice harbors numerous nutrients that are good for health, and has shown potential

antibacterial activity. For example, it has been reported that rice extract can inactivate some pathogenic enteric bacteria, such as *Helicobacter pylori* (Murakami et al., 2005) and *Salmonella enterica* serovar Typhimurium (Kim, Kang, Park, Nam, & Friedman, 2012). There are many different varieties of rice; the most popular is white, but some rice varieties do possess other colors, e.g. brown, red, and black. Little is known about the extraordinary properties of colored rice; therefore, this study aimed to investigate the antibacterial effects of different types of colored-rice crude extract against common bacteria that cause skin and soft tissue infections.

## 2. Materials and Methods

### 2.1 Bacterial Strains and Growth Conditions

All of the bacteria used in this study were kindly provided by the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Thailand. *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922 were used as control strains. The clinical isolates of methicillin-sensitive *S. aureus* (MSSA, no.1-no.8), methicillin-resistant *S. aureus* (MRSA, no.9-no.10), *S. pyogenes* (no.11-no.15), *Enterococcus* spp. (no.16-no.20), *P. aeruginosa* (no.21-no.25) as well as *E. coli* (no.25-no.30) were included in the experimental study, which were independently isolated from infected skin and wound areas.

Bacteria were typically cultured in Luria-Bertani (LB) medium and grown at 37°C with or without shaking for 18-24 h.

### 2.2 Preparation of Rice Extracts

Four kinds of colored rice were obtained from farmers in Chiang Rai Province, Thailand: black non-waxy rice (local name Hom Nil; HN), black glutinous rice (local name Neaw Dum; ND), red rice (local name Mun Poo; MP), and brown rice (local name Sang Yod; SY). The crude extraction process was performed according to the Official Methods of Analysis of the Association of Official Analytical Chemistry (AOAC, 1995) with some modifications. Briefly, one hundred grams of each grain sample was processed by eliminating lipid interference components with n-hexane (500 ml), followed by Soxhlet extraction with ethanol absolute (Merck) for 6 h at a condensation rate of 2-3 drops/sec. The prepared crude extract was then evaporated in an evaporator to remove the solvent. Dry crude extract was resuspended with sterile LB medium and stored at -20°C for analysis.

### 2.3 Antibacterial Susceptibility Testing

To determine the susceptibility of bacteria to rice crude extract, Bauer-Kirby paper disc diffusion/inhibition testing, with some modifications, was used. Each bacterial strain culture had been adjusted to 10<sup>8</sup> colony-forming units (CFU)/ml by measuring optical density (OD) at a wavelength of 600 nm to 0.1. 100 µl of prepared bacterial culture were spread onto a 20 ml Mueller-Hinton (MH) agar plate and left for 5 minutes to dry. Meanwhile, an aliquot of 10 µl of 1 mg/ml of each crude extract was individually applied to sterile filter-paper discs (Whatman no. 1; 6 mm in diameter). Then, the paper discs with 10 µg of rice crude extract were placed onto a bacteria-inoculated MH agar plate and incubated at 37°C. For reagent control testing, the paper disc contained LB medium was also included to show that it did not involve with antibacterial effect. Antibacterial activity was evaluated by measuring the diameter of the zone of growth inhibition around the disc after 24h incubation.

### 2.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

A modified Clinical and Laboratory Standard Institute broth-microdilution method (CLSI, 2009b) was used to obtain the MIC and MBC of the rice crude extracts. Two-fold serial dilutions of each extract were processed to obtain concentrations ranging from 125 to 1000 µg/ml. The bacterial inoculums (100 µl) containing 10<sup>8</sup> CFU/ml were added to each well. A positive control with 1% DMSO and a negative control without an added inoculum were included. The microliter plates were then incubated at 37°C for 18 h. The MIC values were recorded in duplicate as the lowest concentration of rice crude extract that completely inhibited bacterial growth. Minimum bactericidal concentrations were assessed using a selected concentration of crude rice extract that gave significant MIC values and sub-culturing on fresh MH agar. No colony growth was observed.

### 2.5 Assessment of Bacterial Survival

Bacterial survival in the presence of rice crude extracts was noted. In summary, an overnight culture of bacteria was adjusted to OD<sub>600</sub> 0.5 and inoculated at a ratio of 1:500 into standard LB broth with or without additional rice crude extract (to reach a final concentration of 0.5 mg/ml). Every 4 h after inoculation, the cultures were serially diluted for plating and incubated at 37°C for 18-24 h. Bacterial survival was evaluated by counting the CFU. Percent bacterial survival was determined using the formula:

$$\% \text{ Survival} = [\text{CFU}_{(\text{with rice extract})} / \text{CFU}_{(\text{without rice extract})}] \times 100$$

Thus, the bacterial condition without the addition of crude rice extract represented 100% survival.

### 2.6 Statistical Analysis

All assays were conducted in triplicate and Student's *t*-tests of independent experiments were performed using PASW Statistics 18 software. Differences were considered significant for *p* value < 0.05 (\*), < 0.01 (\*\*) and < 0.001 (\*\*\*)

### 3. Results and Discussion

We evaluated the potential antibacterial activity of rice crude extracts from four different types of colored rice (HN, ND, MP and SY) against pathogenic bacteria-causing SSTIs. The antibacterial effects of colored-rice crude extract on control strains of three skin and wound bacteria (*S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922) are shown in Figure 1. Among the control groups, all four colored-rice crude extracts produced significant zones of inhibition only against *S. aureus* ATCC 25923, with no zone of inhibition against *P. aeruginosa* ATCC 27853 or *E. coli* ATCC 25922. The average values of the inhibition zones for the colored-rice crude extracts are shown in Table 1. The results show antibacterial activity against *S. aureus* ATCC 25923 of the colored-rice crude extracts using the disc diffusion assay, with inhibition zones ranging from 7 to 13 mm. The HN crude extract exhibited the strongest antibacterial effect against *S. aureus* ATCC 25923. The MIC and MBC values of the colored-rice crude extracts against the control organisms were also examined. The MICs of the colored-rice crude extracts for *S. aureus* ATCC 25923 were 125 µg/ml for HR, and 250 µg/ml for MP, ND, and SY (Table 2). All colored-rice crude extracts showed anti-staphylococcal action with MBC values at concentrations of 500 µg/ml (Table 2). On the other hand, none of the four colored-rice crude extracts inhibited the growth of *P. aeruginosa* and *E. coli* at any concentration (Table 2). This is the first report of colored-rice extract having an inhibitory effect on *S. aureus*.



Figure 1. Antibacterial activity of four colored-rice extracts against control strains of *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922, by filter disc diffusion method. Filter-paper disc, 6 mm in diameter

Table 1. Inhibition zones of four colored-rice extracts against control strains of *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922 by filter disc diffusion method

Bacteria	Inhibitory zone (mm)			
	HN	MP	ND	SY
<i>S. aureus</i>	9.4±0.5	8.2±0.5	8.5±0.4	8.6±0.4
<i>P. aeruginosa</i>	NZ	NZ	NZ	NZ
<i>E. coli</i>	NZ	NZ	NZ	NZ

Disc size = 6 mm; NZ = No inhibition zone.

Table 2. MIC and MBC of four colored-rice extracts against control strains of *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922

Bacteria	MIC/MBC ( $\mu\text{g/ml}$ )			
	HN	MP	ND	SY
<i>S. aureus</i>	125/500	250/500	250/500	250/500
<i>P. aeruginosa</i>	NA	NA	NA	NA
<i>E. coli</i>	NA	NA	NA	NA

NA = Not applicable.

We also analyzed percentage survival of *S. aureus* ATCC 25923 post-exposure to 500  $\mu\text{g/ml}$  of rice crude extract. Figure 2 shows that, with all colored-rice crude extracts, bacteria survival reduced. Percent survival after colored-rice crude extract treatment reduced significantly, albeit gradually, over time ( $p$  value  $\leq 0.01$ ). About 50% died at 8 h post-exposure to colored-rice crude extract. Among the four extracts, HN exhibited antibacterial activity with the greatest reduction in *S. aureus* survival. In contrast, MP exhibited antibacterial activity with the smallest reduction in *S. aureus* survival. However, ND and SY showed similar declines in antibacterial activity against *S. aureus*. These results indicated that different colored-rice extracts have different levels of antibacterial activity.

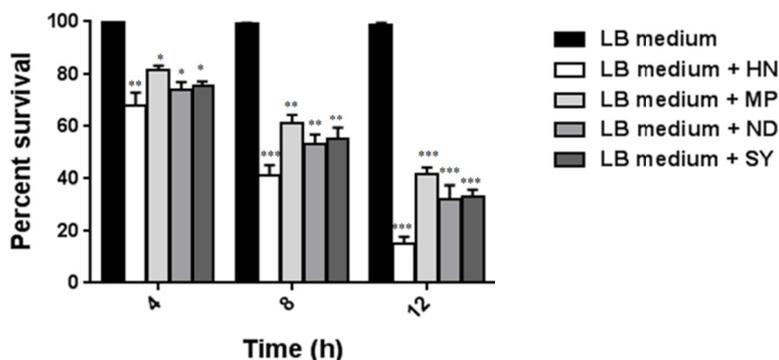


Figure 2. Evaluation of *S. aureus* survival in the presence of colored-rice extracts. Overnight culture of *S. aureus* ATCC 25923 was inoculated in five differential media (LB broth with or without 500  $\mu\text{g/ml}$  colored-rice extracts) and maintained at 37°C. Their viable colony-forming units (CFU) were counted before and after 4, 8, and 12 h of treatment with colored-rice extract and calculated for percent survival, as explained in the Materials and Methods section. Data shown are averages from three experiments. Asterisks indicate significant differences between the LB-broth, and LB broth with 500  $\mu\text{g/ml}$  colored-rice extract, groups. \* represents  $p$  value  $< 0.05$ , \*\* represents  $p$  value  $< 0.01$  and \*\*\* represents  $p$  value  $< 0.001$

Colored-rice crude extracts were further investigated against a panel of clinically isolated skin and wound bacteria (MSSA, MRSA, *S. pyogenes*, *Enterococcus* spp., *P. aeruginosa*, and *E. coli*). Table 3 shows the sensitivity of MSSA- and MRSA- clinical isolates to all four extracts, with inhibition zones ranging from 7 to 11 mm. As expected, HN extract showed the greatest inhibitory activity against both strains of clinical *S. aureus*, like control strain ATCC 25923. Moreover, all colored-rice extracts showed MIC and MBC against clinically isolated *S. aureus* at concentrations of 125-500  $\mu\text{g/ml}$  and 500-1000  $\mu\text{g/ml}$ , respectively (Table 4). Conversely, clinical isolates of *S. pyogenes*, *Enterococcus* spp., *P. aeruginosa* and *E. coli* were resistant to all colored-rice crude extracts, as observed with control strains of *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922. Taken together, there was better activity against *S. aureus* than other bacteria, indicating colored-rice extracts had specificity for antibacterial activity.

Table 3. Inhibition zones of four colored-rice extracts against clinical isolates of *S. aureus*, *S. pyogenes*, *Enterococcus* spp., *P. aeruginosa*, and *E. coli*, by filter disc diffusion method

Clinical isolate		Inhibitory zone (mm)			
Bacteria	No.	HN	MP	ND	SY
<i>S. aureus</i>	1	9.4±0.3	8.3±0.5	8.5±0.4	8.6±0.4
	2	9.1±0.5	8.2±0.7	8.6±0.5	8.5±0.3
	3	9.2±0.2	8.2±0.4	8.4±0.3	8.5±0.3
	4	8.7±0.4	7.8±0.3	8.2±0.2	8.2±0.4
	5	8.9±0.2	8.2±0.3	8.3±0.4	8.4±0.2
	6	9.0±0.4	8.2±0.5	8.4±0.5	8.4±0.5
	7	8.8±0.5	8.2±0.4	8.2±0.4	8.2±0.4
	8	8.6±0.5	7.9±0.4	8.2±0.4	8.3±0.4
	9	7.4±0.5	6.4±0.5	6.8±0.5	6.8±0.5
	10	7.2±0.5	6.4±0.5	6.8±0.5	6.8±0.5
<i>S. pyogenes</i>	11-15	NZ	NZ	NZ	NZ
<i>Enterococcus</i> spp.	16-20	NZ	NZ	NZ	NZ
<i>P. aeruginosa</i>	21-25	NZ	NZ	NZ	NZ
<i>E. coli</i>	26-30	NZ	NZ	NZ	NZ

Disc size = 6 mm; NZ = No inhibition zone.

Table 4. MIC and MBC of four colored-rice extracts against clinical isolates of *S. aureus*, *S. pyogenes*, *Enterococcus* spp., *P. aeruginosa*, and *E. coli*

Clinical isolate		MIC/MBC (µg/ml)			
Bacteria	No.	HN	MP	ND	SY
<i>S. aureus</i>	1	250/500	250/500	250/500	250/500
	2	125/500	250/500	250/500	250/500
	3	250/500	250/500	250/500	250/500
	4	500/500	500/500	500/500	500/500
	5	250/1,000	500/1,000	500/1,000	500/1,000
	6	250/500	250/500	250/500	250/500
	7	250/500	250/500	250/500	250/500
	8	250/500	250/500	250/500	250/500
	9	500/1,000	500/1,000	500/1,000	500/1,000
	10	500/1,000	500/1,000	500/1,000	500/1,000
<i>S. pyogenes</i>	11-15	NA	NA	NA	NA
<i>Enterococcus</i> spp.	16-20	NA	NA	NA	NA
<i>P. aeruginosa</i>	21-25	NA	NA	NA	NA
<i>E. coli</i>	26-30	NA	NA	NA	NA

NA = Not applicable.

With the increase in resistance to many existing antibacterial agents (Doern, et al., 1999; Jones, et al., 2004; Liu, 1999), especially in the treatment of skin diseases, there is a good opportunity to develop new treatments using plants or plant-based formulas. Many plants are already commonly used in several countries to treat or prevent SSTIs (Dholvitayakhun, Cushnie, & Trachoo, 2012; Mahady, 2005; Olugbuyiro, Abo, & Leigh, 2010;

Trakulsomboon, Kummalue, & Jiratchariyakul, 2006). Nevertheless, limited data currently exist to prove their efficacy against SSTI-related pathogens. Rice (*Oryza sativa* L.) has recently become an interesting candidate as a natural antibacterial agent. It has been reported that rice extract is effective in suppressing damage to the gastric mucosa in *H. pylori*-infected Mongolian gerbils (Murakami, et al., 2005). Moreover, rice-hull-smoke extract has been shown to inhibit *S. enterica* serovar Typhimurium *in vitro*, and protected infected mice from death (Kim, et al., 2012). This study has substantiated the antibacterial property of colored-rice extracts, in particular against skin and wound pathogenic *S. aureus*. The antibacterial activity of colored-rice extracts was examined using several reliable techniques. It has been noted that Bauer-Kirby disk susceptibility-, MIC- and MBC-based determination are widely used for investigating the antimicrobial susceptibility of various bacteria (Gonzalez et al., 2013; Liberman & Robertson, 1975; Traub, Spohr, & Bauer, 1987; Van Asselt & Van Boven, 1997).

Colored rice varieties-mainly black, red, and purple-contain a variety of bioactive constituents, i.e. flavones, anthocyanins, tannin, phenolics, sterols, tocopherols,  $\gamma$ -oryzanol, amino acids, and essential oils (Chakuton, Puangpronpitag, & Nakornriab, 2012; Deng et al., 2013). Several phenolic compounds of colored-rice have been reported on the antioxidant and anti-inflammatory properties, both *in vitro* and *in vivo* (Fujita, Fujitake, Kawakami, & Nomura, 2010; Muntana & Prasong, 2010; Toyokuni et al., 2002). However, the antimicrobial effect of colored rice has not been reported before. Presently, our study results strongly suggested that colored-rice crude extracts exhibited anti-staphylococcal activity. However, larger sample sizes and other varieties of SSTI-causing bacteria, as well as a wider range of colored-rice varieties are required for further investigation, to provide a better understanding of specific inhibition. Although colored-rice crude extracts may not affect some bacteria, such as *S. pyogenes*, *Enterococcus* spp., *P. aeruginosa* and *E. coli*; they could be used to develop medical formulas in combination with other antibacterial agents with proven effect on these bacteria.

#### 4. Conclusion

Our findings exhibited a basis for future applications of colored-rice crude extracts, which may provide advantages in skin treatment or protection against bacterial infections. For example; incorporating colored-rice crude extracts into medicines and/or skin-care products, such as soaps, creams, and lotions, to protect the skin from bacterial infections. However, further intensive research in animals and humans is required, to verify the safety, efficacy, and mechanism of action of colored-rice extracts in the prevention and treatment of infections caused by bacterial pathogens.

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